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LACK OF Mecp2 INTERFERES WITH CORTICAL PROGENITORS PROLIFERATION AND DIFFERENTIATION

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Abstract

Rett syndrome (RTT) is a progressive neurological disorder affecting 1 in 10.000 live female births; nowadays is the most common genetic cause of intellectual disability in females. RTT is characterized by a normal pregnancy period and an apparently normal early post-natal phase. Around 6-18 months of life children lose all the previously acquired skills and manifest overt symptoms (as autistic features, loss of purposeful hand use, respiratory abnormalities and motor dysfunctions). Classical RTT cases are linked to mutations in the X-linked *MECP2* gene that encodes for the Methyl-CpG-Binding Protein 2 (MeCP2), a multi-functional protein ubiquitously expressed in the body.

The apparently normal early post-natal period explains why most of the studies in the RTT field focused post-natally, while the role of the protein during early development was only partially investigated. However, nowadays, many evidences demonstrating the presence of earlier symptoms in both girls and RTT animal models are arising. Therefore, we decided to investigate the role of Mecp2 during early/embryonic cortical development, as the cerebral cortex morphology is altered in RTT and patients manifest cognitive impairments. We demonstrated that E15.5 cortices are characterized by an altered expression profile suggestive of a delay in neuronal maturation. Thus, we analysed neuroprogenitors highlighting that Mecp2 absence slightly alters the cell cycle progression, lengthening the permanence of cells in the G1 phase. This possibly impairs the proper definition of cell fate, as *Mecp2* null embryonic cortices are characterized by the increment of cells transitioning from progenitors to post-mitotic neurons. Interestingly, the altered switch is in line with the maturation delay suggested by our transcriptional analysis, as the impaired fate definition can delay the expression of genes characterizing more mature cells as different glutamate receptors and ion channels subunits. Cells, delayed in their maturation since early/embryonic phases, are not properly responding to external stimuli, resulting in their defective integration in the forming network, which is a feature common to many autistic spectrum disorders. Our data represent a first evidence of the importance of Mecp2 during embryonic development and suggest that the phenotype characterizing RTT patients is due to the sum of different alterations occurring since embryonic development and worsening through life.

1. INTRODUCTION

1.1 Rett syndrome

It was 1966 when Dr. Andreas Rett published a first description of a syndrome characterizing different young females all in paediatric ages and showing common features and unusual behaviours. Unfortunately, the pathology remained unknown for at least other 20 years, probably due to its rarity and to the fact that Dr. Rett published exclusively in the German medical literature. The description of a larger group of girls affected by Rett syndrome (named in honour of Dr. Rett; RTT; OMIM #312750) was published in English almost twenty years later, bringing the pathology to be internationally recognized (Chahrour and Zoghbi, 2007; Lombardi et al., 2015). RTT is a progressive neurological disorder affecting 1 in 10.000 live female births.

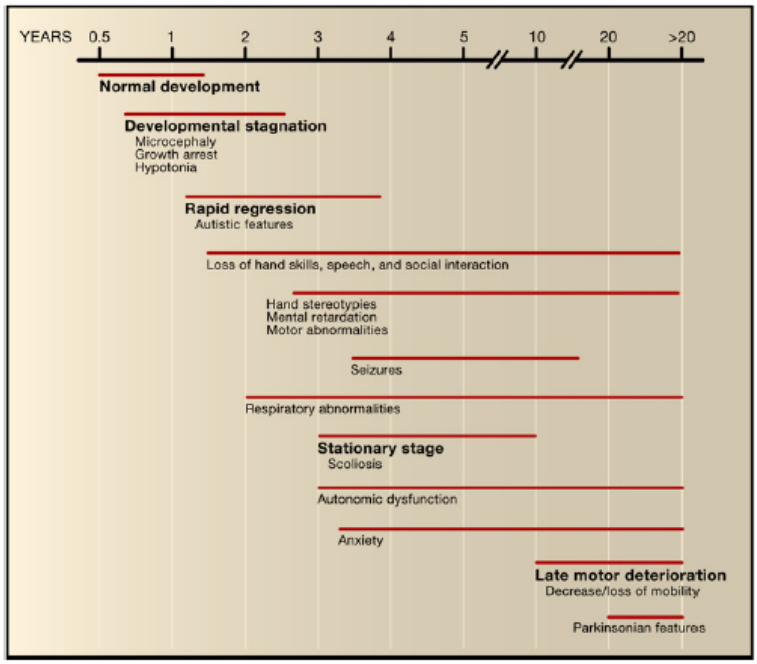

Figure 1.1 Onset and progression of clinical symptoms of RTT. Patients affected by RTT go through a common progression of symptoms. They usually are normal at birth, but then enter in a phase of developmental stagnation, followed by a rapid regression with the manifestation of autistic features, a stationary phase and finally a late motor deterioration (Chahrour and Zoghbi, 2007).

Nowadays RTT is the most common genetic cause of severe intellectual disability in females (Gadalla et al., 2011); its severity and clinical presentation can be widely variable but a cascade of symptoms common to most of the patients can be recognized (Fig 1.1).

RTT is characterized by a normal pregnancy period followed by an early post-natal phase without symptoms; importantly, considering this phase as completely symptoms-free is incorrect, as many retrospective analysis showed that hypotonia is already present in the "pre-symptomatic" phase and development in general present subtle, but consistent, alterations (Smeets et al., 2012). Between 6 and 18 months of age, a phase of *developmental stagnation* is usually recognizable. This stage is characterized by (i) the inability to acquire different developmental milestones (as word development, social interaction and motor functions), (ii) the delay of growth, (iii) the appearance of microcephaly and (iv) strong muscle hypotonia, generating the so-called "weak posture". Afterwards, the pathology rapidly develops with the *deterioration period*, in which any previously acquired milestone is lost and other symptoms appear. If children were walking or standing, speaking or babbling they generally lose these capabilities; moreover they start showing strong autistic features as social withdrawal, irritability, self-abusive behaviour and indifference to the surrounding environment together with one of the classical features of RTT, the lost of purposeful hand use that is replaced by stereotypical movements (usually clasping or hand washing movements). Often, in this phase, patients develop respiratory abnormalities (as hyperventilation or apneas) and motor dysfunctions (as apraxia or ataxic gait). The deterioration period is followed by a *pseudo-stationary phase*, that starts between 3 and 10 years of age, in which usually social interaction and autistic features ameliorate, as children appear more alert and joyful. The improvement is only partial, in fact children in this stage generally develop seizures and scoliosis. The last phase is the so-called *late motor deterioration*; patients often become wheelchair dependent, presenting muscle waste and circulation problems. At this stage the neurological impairment become even severer and patients may develop Parkinsonian features (Chahrour and Zoghbi, 2007; Lombardi et al., 2015).

1.2 The genetic of RTT

Since the first descriptions of RTT it was evident that the pathology is mostly affecting females without prevalence in any racial or ethnic group (Percy and Lane, 2013), leading to the hypothesis that the syndrome was due to a dominant X-linked mutation, permitting survival only of heterozygous females, while resulting lethal for males. Few years later, RTT was linked to mutations of the X-linked *MECP2* gene (Amir et al., 1999), that alone explain more than 95% of classic RTT cases.

Due to the severity of symptoms, usually, patients do not reproduce, thus the syndrome is sporadic in 99% of cases; few exceptions are represented by mechanisms of skewed X chromosome inactivation (which is normally around 50% for each allele, while in these cases could be unbalanced 70-80% towards the mutated allele) that generates girls without strong symptoms, due to a "dilution" of the mutated allele. Unfortunately, these girls may reproduce generating children that inherit the mutation, but not the pattern of X chromosome inactivation, thus manifesting the syndrome with its common progression (Lombardi et al., 2015).

To further explain the preponderance of feminine cases in RTT, it was demonstrated that generally the disease-causing mutations occur mainly in the paternal germline, from which females inherit directly one of the X chromosomes. More rarely, when the mutation is in the maternal germline, it could lead to the classical RTT in females or to a much more severe condition in males, which usually causes death in the first two years of life and often is not even recognized as RTT, but generally defined as encephalopathy. In few cases, males manifest a form of RTT compatible with life when inheriting a mutation that do not generate a complete loss of function of the protein or in a Klinefelter (XXY) background with one of the two X chromosome mutated (Lyst and Bird, 2015; Trappe et al., 2001).

The diagnosis of RTT can be difficult due to the variety of symptoms; nowadays, it is based on internationally accepted diagnostic criteria (Smeets et al., 2012) followed by molecular genetic testing. Importantly, the genetic alteration of *MECP2* alone, even if necessary, is not sufficient for the RTT diagnosis, as *MECP2* modifications are known to be present in a large group of cognitive deficits (like Angelman-like

syndrome, nonsyndromic mental retardation and schizophrenia) (Percy and Lane, 2013; Smeets et al., 2012).

1.3 The Methyl-CpG-binding Protein 2 (MeCP2)

MECP2 is located on the X chromosome (region q28) and consists of 4 exons occupying a region of 76 kb, encoding for the Methyl-CpG binding protein 2 (MeCP2). Due to alternative splicing, two different isoforms of the protein are generated: MeCP2-e1 is the longer one and contains 21 unique N-terminal amino acids, is encoded starting from the exon1 and skipping 9 residues on the exon 2 (498 aa); MeCP2-e2 is encoded starting from the exon 2 and contains 9 unique amino acids (486 aa) (Fig. 1.2). The two isoforms are considered functionally equivalent, as all the known *MECP2* mutations are in the common region, but the isoform e1 is predominantly expressed in the brain (Bedogni et al., 2014; Kerr et al., 2012).

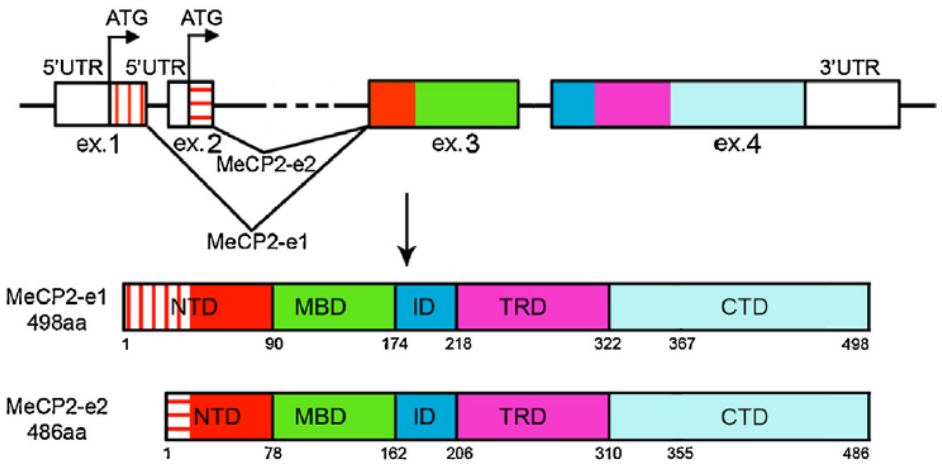

Figure 1.2 *MECP2* structure and splice variants. The *MECP2* gene is formed by 4 exons coding for two different MeCP2 variants. MeCP2-e1 is the longest one (498aa) and is transcribed starting from exon 1 and skipping exon2. MeCP2-e2 transcription (486aa) starts from exon 2 (Bedogni et al., 2014).

As depicted in figure 1.3, the protein can be divided in 5 structural domains: the N-terminal Domain (NTD, 1-78 aa), the Methyl-CpG Binding Domain (MBD, 79-162 aa), the Intervening Domain (ID, 163-206 aa), the Transcription Repression Domain

(TRD, 207-310) and the C-Terminal Domain (CTD, 311- 355 aa CTDα and 356-486 CTDβ). Moreover, other functional domains were identified (Fig. 1.3) including the Nuclear Localization Signal (NLS, within the TRD), the WW domain (involved in splicing regulation) and two PEST sequences (involved in rapid proteolytic degradation) (Bedogni et al., 2014).

Figure 1.3 MeCP2 can be divided in different structural and functional domains. MeCP2 structural domains are: the N-terminal Domain (NTD), the Methyl-CpG Binding Domain (MBD), the Intervening Domain (ID), the Transcription Repression Domain (TRD) and the C-Terminal Domain (CTD). The MeCP2 functional domain include: the Nuclear Localization Signal (NLS), the WW domain and two PEST sequences (Bedogni et al., 2014)

MeCP2 was discovered in 1992, through biochemical studies, as a protein that binds methylated cytosine (5mC) belonging to CpG dinucleotides (Lewis et al. 1992); because of its high abundance in brain it is globally bound across the genome (mainly following the distribution of the 5mC; (Skene et al., 2010)). This highly specific binding is mediated by the MBD which, interestingly, recognizes the methylation-dependent hydration of the major groove of the DNA, rather than the cytosine methylation itself (Bedogni et al., 2014). MeCP2 binding to the DNA is far more complicated than recognizing exclusively methylated DNA, even if the functional meaning of the different bindings still needs to be clarified. Indeed, in the brain, it also binds the 5-hydroxymethylcytosine (5hmc) through the MBD (Mellén et al., 2012). Moreover, it was demonstrated that MeCP2 contains unspecific binding sites (in the ID, TRD and CTD) for unmethylated DNA (Ghosh et al., 2010); it is possible that this binding just facilitates the association with methylated CpG (Hansen et al., 2010). Recently, it was demonstrated that MeCP2 also interacts with CpA dinucleotides; interestingly, this modification is prevalent in neurons and accumulates in the same period in which MeCP2 levels drastically increase during development, possibly, massively recruiting the protein to chromatin (Guo et al., 2014).

Until today, hundreds of RTT pathogenic mutations have been identified. Interestingly, only eight point mutations justify almost 70% of RTT cases, the others

are explained by small C-terminal deletions or complex chromosomal rearrangements. Generally, mutations affecting the NLS or the MBD are associated with a severer form of the pathology, while mutations affecting the C-terminal region give milder symptoms. Highlighting the importance of the MBD function, roughly half of the missense mutations causing RTT are in this domain and impair its binding specificity. Thanks to the MBD, the protein localizes mainly at pericentromeric heterochromatin (containing highly methylated DNA); if the MBD looses its capacity to bind 5mC the protein appears diffused into the nucleus. Although less frequent, many RTT causing mutations occur in the CTD or the ID, therefore highlighting also the relevance of these domains for the correct function of the protein (Bedogni et al., 2014; Bellini et al., 2014; Chahrour & Zoghbi, 2007).

1.4 MeCP2: a multifunctional protein

MeCP2 has a role in many different cellular processes, possibly explaining the huge variety of symptoms generated by its absence (Fig. 1.4). The MeCP2 structure itself suggests that it is a multifunctional protein. Indeed, only the MBD seems to have a defined structure, while almost 60% of the protein is unstructured and may assume different conformations interacting with different molecules that modify or regulate its activity (Bedogni et al., 2014).

Given the presence of the TRD (the smallest portion of the protein indispensable to have transcriptional repression *in vitro*), one of the first roles attributed to Mecp2 was as *transcriptional repressor*. Indeed, it was demonstrated that Mecp2 interacts with co-repressor complexes (such as SIN3A (Nan et al., 1998) or NCoR-SMRT (Stancheva et al., 2003)) containing histone deacetylase (HDAC) activities. The recruitment on the DNA of chromatin remodelling factors (such as HDAC) led to the hypothesis that Mecp2 exerts its repressive function through *chromatin structure compaction* rather than working as a classical transcription factor, which target specifically single genes. Indeed, it was demonstrated that, in neurons, where Mecp2 is highly abundant, it binds at the level of the linker DNA and it is able to displace H1, condensing the chromatin structure and globally reducing transcription. Reinforcing this theory, neurons present high levels of Mecp2, but only half of the

quantity of histone H1 compared to other cellular types, suggesting that Mecp2 could functionally substitute this linker histone. Accordingly, it was demonstrated that neurons lacking Mecp2 present defects at the level of higher-order chromatin structure, increased levels of histone H1 and of histone acetylation (Kernohan et al., 2014; Lombardi et al., 2015; Skene et al., 2010). Moreover, the absence of Mecp2 in neurons generates an elevated transcriptional noise due to the lack of repression of repetitive sequences and L1 retrotransposons.

The fact that these effects are neuron specific suggests that Mecp2 function depends on its abundance (Muotri et al., 2010; Skene et al., 2010).

Further complicating the role of Mecp2, some data suggest that MeCP2 might also *activate transcription*. Indeed, Chahrour et al reported that cells lacking Mecp2 present more down-regulated genes than up-regulated; the authors suggested that these activating functions are mediated by the capability of Mecp2 to recruit on the DNA the transcriptional factor CREB1 (cyclic AMP-responsive element binding protein 1) (Chahrour et al., 2008). The capability of MeCP2 to bind 5hmC, an epigenetic signal associated with gene activity (Mellén et al., 2012), further suggests a role for Mecp2 in transcriptional activation. Thus, the precise role of MeCP2 in regulating transcription remains badly defined, and it is highly possible that it depends on the gene locus bound and on its interactions (Bedogni et al., 2014; Lyst & Bird, 2015).

In addition it has been proposed that Mecp2 may regulate gene expression either through its capability to bind RNA (Jeffery and Nakielny, 2004) or through its interaction with proteins involved in splicing such as YB1 (Y box-binding protein; a transcription factor regulating alternative splicing (Young et al., 2005)) and Prpf3 (pre-mRNA processing factor 3; a spliceosome associated protein; (Long et al., 2013)). Since Mecp2 deficiency causes the up regulation of different miRNAs, a role in miRNA processing has also been proposed (Cheng et al., 2014); this function is probably occurring through the interaction of Mecp2 with DGCR8 (DiGeorge Syndrome Critical Region 8; subunit of the complex mediating the biogenesis of miRNAs), impeding its communication with Drosha.

Finally, it was demonstrated that Mecp2 regulates the protein synthesis. Indeed, its absence causes the reduction of the AKT/mTOR signaling significantly impairing

translation (Ricciardi et al., 2011), while the enhancement of the protein synthesis ameliorates the phenotype of neurons lacking Mecp2 (Y. Li et al., 2013).

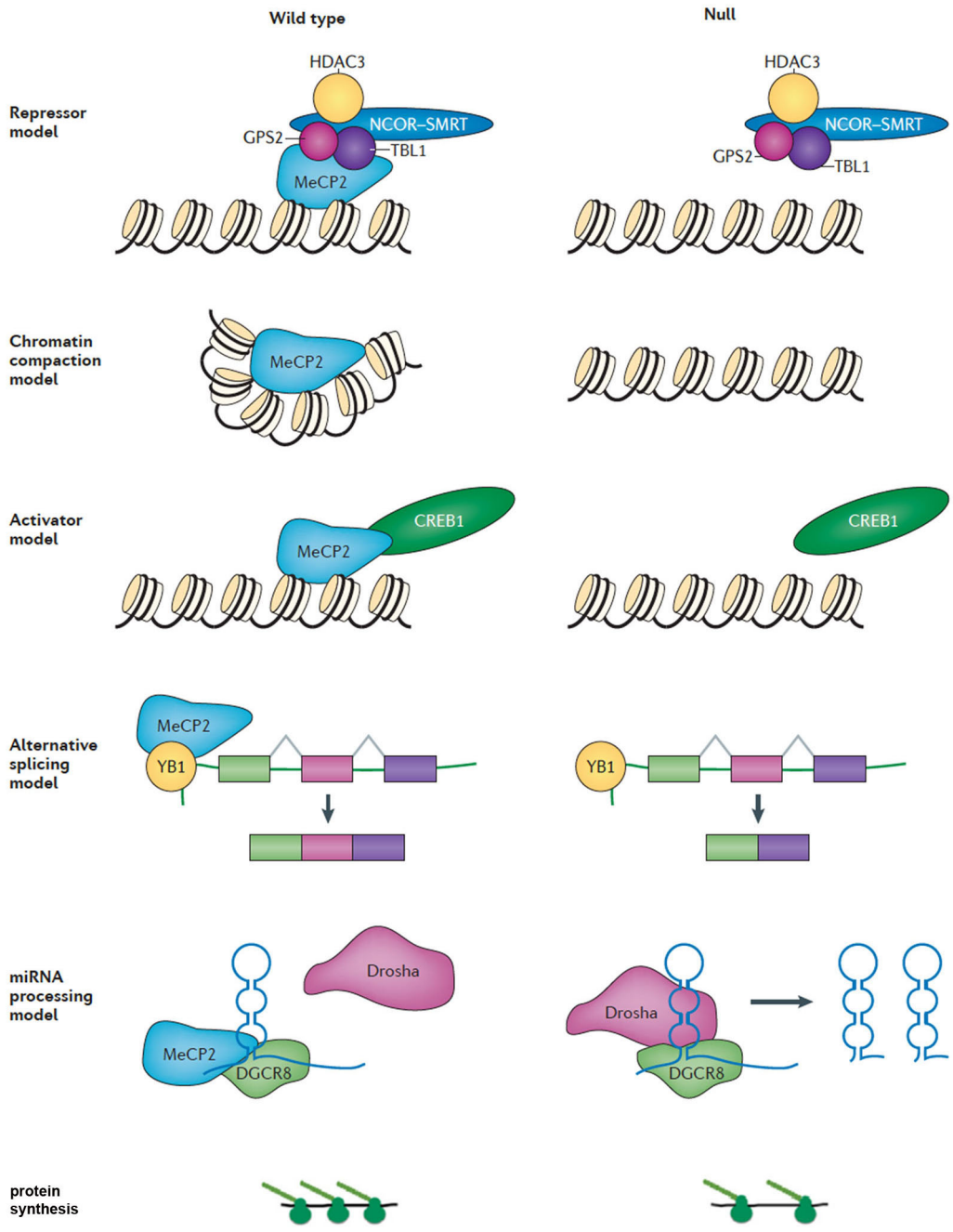

Figure 1.4 MeCP2 as a multifunctional protein. Through years many different functions of MeCP2 have been discovered. Nowadays the most accepted functions of the protein are involved in: chromatin compaction, repression and activation of transcription, alternative splicing, miRNA processing and protein synthesis (modified from Lombardi et al. 2015).

It is generally accepted that MeCP2 functions are also tuned by different types of post-translational modifications (PTM) that modify its binding to DNA/chromatin and/or its interaction with other molecules. Indeed, MeCP2 can be phosphorylated, acetylated, methylated, ubiquitinated and sumoylated; of note, the significance of most of these modifications is still unknown. Phosphorylation represents the PTM of MeCP2 best characterized; it can occur at different levels and can affect gene expression, adaptation to stimuli and interaction with partners (Bellini et al., 2014). The first evidence of Mecp2 phosphorylation was on serine 421 (S421) and it was linked to neuronal activity. Indeed, it was demonstrated that Mecp2 normally sits on the BDNF promoter and, as a consequence of neuronal membrane depolarization, is phosphorylated on S421; the phosphorylation event causes the detachment of the protein from the promoter and the consequent transcription of *Bdnf* (Brain-Derived Neurotrophic Factor). Interestingly, this modification is neuronal specific and, through the use of knock in mouse lines bearing a phosphodefective mutation in the S421 site (S421A), it was demonstrated that it is important for the dendritic growth and the development of circuits (Bellini et al., 2014; Chen et al., 2003). Subsequently, it was demonstrated that neuronal activity also induces the phosphorylation of S424 and a knock in mouse line, harbouring the non-phoshporylatable mutations S421A and S424A was generated. These mice do not show any strong phenotype; surprisingly, they seem to perform better in different behavioural tests, confirming that the MeCP2 phosphorylation can affect the functionality of the nervous system (Li et al. 2013). Another well-studied phosphorylation of Mecp2 is the one that occurs on S80. It is typical of resting neurons and gets removed upon neuronal stimulation; functionally, it appears to increase the affinity of Mecp2 for chromatin. Although the phosphorylation of S80 appears as the most abundant, the phosphodefective S80A knock in mouse, generated to study this modification, only manifests a subtle weight increase and a decreased locomotor activity (Tao et al., 2009). The importance of Mecp2 phosphorylation has been recently strengthened by the study of the one on threonine 308 (T308); indeed, the phosphodefective T308A knock in mouse model manifests strong symptoms typical of RTT mice. It was demonstrated that the modification of this residue abolishes the interaction of Mecp2 with NCoR, reducing the

transcriptional repression normally mediated by this complex (Ebert et al., 2013; Moretti et al., 2006). Many more phosphorylated sites have been recently mapped, but the great majority of them have not been characterized yet. We believe that the discovery of pathogenic point mutations on phosphorylation sites will not only highlight the importance of these modifications, but might also lead to novel information on Mecp2 functioning (Bellini et al., 2014). Accordingly, the identification of a RTT patient carrying a phosphomimetic mutation of Tyrosine 120 (Y120D) led to the careful analyse of this phosphorylation site permitting to demonstrate a novel localization and functional association of the methyl binding protein with the centrosome (Bergo et al., 2015).

In conclusion, it is possible that Mecp2 exerts different functions in different cellular types, depending on its abundance, on the genomic area bound, on post-translational modifications and sensing the cellular specific levels and patterns of DNA methylation. Further increasing the complexity of the activity of MeCP2, the different post-translational modifications may be independent one from each other or may combine in a complex cross talk regulating its functions (Bellini et al., 2014).

1.5 Rett syndrome animal models

Great achievements for the comprehension of Mecp2 functions derive from the generation of transgenic mouse models; here we will revise the most significant for the study presented in this work.

In 2001 two independent laboratories produced two different *Mecp2* knock out (ko) animals. The first one (*Mecp2*$^{tm1.1Bird}$) was generated on a C57BL/6 background deleting exons 3 and 4 of the *Mecp2* gene, thus removing most of the coding portion (with the exception of the N-terminal amino acids) (Guy et al., 2001). While, Dr Jeanisch and colleagues produced an animal (*Mecp2*^{Jae}) devoid only of exon 3 (including the great majority of the MBD) on a mixed genetic background (129, C57BL/6 and BALB/c) (Chen et al., 2001). Both these mouse lines are considered valid models for the study of RTT, since they manifest many of the major symptoms characterizing the pathology in humans. Importantly, the two models are quite

similar, presenting an overlapping progression of symptoms and the same life span; thus from now on we will generically talk of the Mecp2 ko.

Importantly, in the RTT field, it is accepted the use of the male ko mouse as the main model of the syndrome. Indeed, the unpredictability of the X chromosome inactivation pattern generates a not reproducible phenotype in heterozygous females. Furthermore, because of the *Mecp2* ko male sterility, mouse females are precious to maintain the colony.

Hemizygous *Mecp2* ko mice appear normal at birth and for the subsequent 3-5 weeks, after which they progressively develop several neurological impairments and die 2-4 weeks later. Symptomatic hemizygous males show reduced movements and weight, abnormal gait, hind limb clasping, tremors and poor general conditions. The expected lifespan is approximately 7-10 weeks (Chen et al., 2001; Guy et al., 2001).

Importantly, as already mentioned, the ko male mice are sterile; thus, is not possible to generate ko females and in order to maintain the colony is necessary to breed heterozygous females with wt males. This mating condition generates only 25% of ko animals per litter that, together with the small number of animals per litter normally produced by C57BL/6 animals, represent a major issue when collecting animals for experimental procedures. Moreover, managing these animals is particularly challenging as the phenotype of null males tends to deteriorate as the colony ages, hardening the detection of symptoms directly ascribable to the absence of *Mecp2* and not to the general bad conditions of the animals. Lastly, heterozygous females are very aggressive and often cannibalize their litter and/or take less care of pups, generating detrimental effects on the phenotype of the progeny. To reduce these difficulties of colony management many studies in the RTT field have been performed on mixed backgrounds. In our laboratory we transferred the *Mecp2*$^{tm1.1Bird}$ genetic modification on the outbred CD1 (ICR) background. We hypothesized that this background could, at least partially, solve the presented issues thanks to the robustness of these animals and to the their tendency to produce large litters (Aldinger et al., 2009). Indeed, the first advantage we observed is the drastic increment of the number of pups per litter (which almost double compared to the C57BL/6 colony), obviously increasing the number of ko animals obtained from single crossings. Interestingly, females take better care of their progeny, as the

number of cannibalized litters is incredibly reduced. Mice were tested to evaluate the development of the typical RTT phenotype (Fig. 1.5) and, as expected, they manifest all the main symptoms (tremors, reduced mobility and hind limb clasping) of the C57BL/6 ko animals, approximately in the same time window. Interestingly, the life span of the CD1 ko is prolonged as the peak of mortality is around P70-80. We concluded that the RTT CD1 mouse is a good model for the pathology and, being easier to maintain and manage compared to C57BL/6 animals, it represents an improvement for the RTT field (Cobolli Gigli et al., under final review).

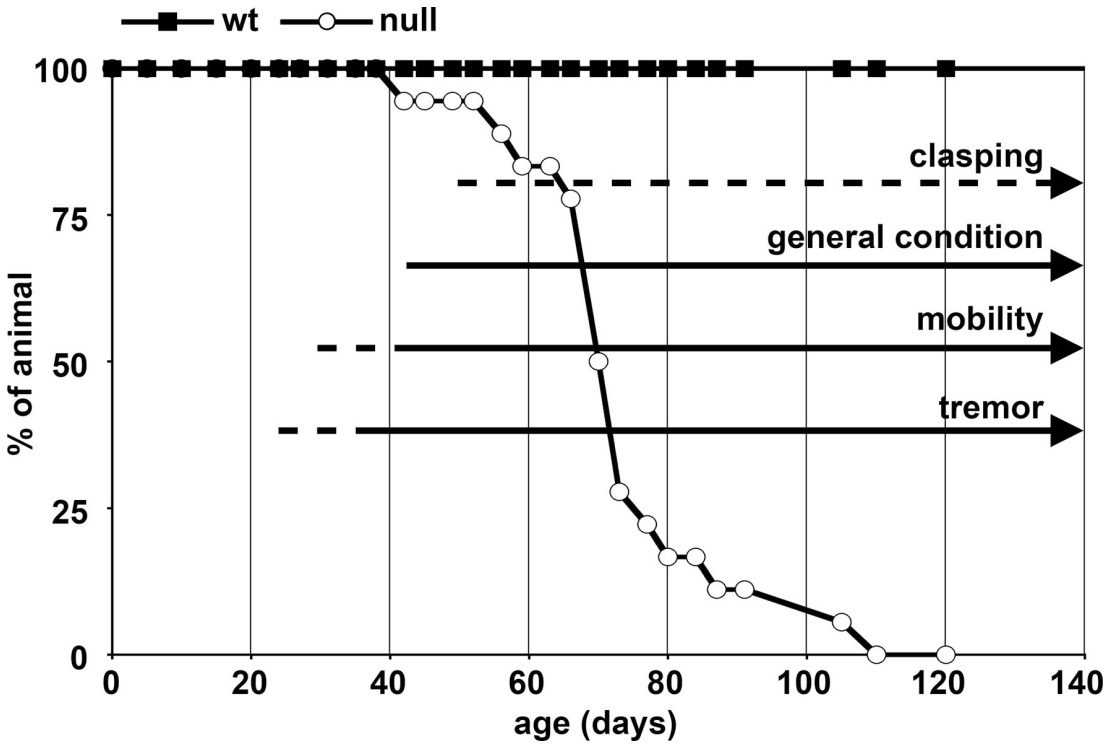

Figure 1.5 The *Mecp2*$^{tm1.1Bird}$ modification was transferred on the CD1 genetic background. The plot represents the average life span of CD1 null animals compared to wt controls. Arrows represent the timing of appearance of the different symptoms typical of the C57BL/6 mice (dotted lines = 50-99% of animals have the given symptom, continuous line = 100% of the animals have the symptom) (Cobolli Gigli et al., under final revision).

1.6 The central role of the brain in RTT pathogenesis

Although Mecp2 expression is ubiquitous in the body, RTT patients and mice are mainly characterized by neurological symptoms, suggesting that the brain may have a central role in RTT pathogenesis. To evaluate the involvement of the central

nervous system in the development of the phenotype, different groups produced mouse models, lacking Mecp2 expression exclusively from the brain, exploiting the Cre/loxP technology. At first, Mecp2 expression was removed specifically from neurons and glial cells, since their precursor stage (Nestin positive cells), obtaining a brain completely devoid of Mecp2 expression starting from the embryonic day 10. Interestingly, the phenotype of these mice resulted mainly undistinguishable from the one of the *Mecp2* total ko animals, demonstrating for the first time that RTT symptoms are mainly ascribable to the lack of Mecp2 from the brain (Chen et al., 2001; Guy et al., 2001).

Subsequently, given that children were considered normal at birth and symptoms became evident after 6-18 months of life, it was evaluated whether the deletion of Mecp2 only from post-natal neurons was sufficient to cause the phenotype. A mouse model in which Mecp2 expression is ablated only in CamkIIα positive neurons was thus generated; CamKIIα starts to be expressed postnatally at low levels (P1) and its levels considerably increase with neuronal maturity, meaning that Mecp2 would be correctly expressed throughout the entire embryonic development. Interestingly, these mice manifested a phenotype similar to the one of the total ko mice, but delayed (starting around 3 months of life) and less severe. This result demonstrated that Mecp2 has a role in postnatal neurons, as the removal of it from these cells generates a RTT-like syndrome. Moreover, the syndrome is mainly ascribable to the removal from neurons rather than from glial cells, that in this model correctly express Mecp2 (Chen et al., 2001).

Surprisingly, given the proved central role of the brain in RTT pathogenesis and the severity of the neurological symptoms developed by RTT patients, there are no big impairments in the morphology of the brain. Blood vessels, cranial nerves, gyri, basal ganglia, cerebellum, brain stem and spinal cord are not altered and there are no signs of degeneration or inflammation. The main characteristic of the RTT brains is the reduced head circumference, due to a decrement in brain size and weight, with a particularly evident effect in the prefrontal, posterior frontal and anterior temporal regions. The diminished dimension of the brain is ascribable to a reduction of the neuronal soma size and to an increment in cell density, while the total number of neurons does not change. Moreover, RTT brains are characterized by decreased

spine density and dendritic arborisation in both the hippocampus and the cortex; in some cases hypoplasia of corpus callosum was also observed (Armstrong et al., 2003; Bauman et al., 1995; Kishi and Macklis, 2004).

The morphology of the RTT brain was further and deeply investigated using animal models. Most of the impairments recognized in RTT patients were also found in the *Mecp2* hemyzigous male mice. Indeed, these animals are characterized by a reduced thickness of the cerebral cortex (roughly 20% less than wt controls), due to the diminished neuronal soma size and the increased cellular density. Moreover, the dendritic arborisation is poorly developed, with a reduction of the number of spines and synapses both in young "pre-symptomatic" and adult symptomatic mice (Belichenko et al., 2009; Chao et al., 2007; Fukuda et al., 2005; Kishi and Macklis, 2004). Importantly, the impairments in synaptic formation and maturation may lead to alterations in cerebral plasticity. Thus, many groups evaluated the correct functioning of impulses transmission and synaptic plasticity in order to link structural phenotypes to changes in physiology, possibly explaining some RTT features. It was demonstrated that pyramidal neurons, deriving from the layer 5 of the somatosensory cortex of *Mecp2* ko animals, display reduced spontaneous activity at both "pre-symptomatic" (second postnatal week) and early symptomatic stages. Moreover, RTT brains are characterized by the unbalance between cortical excitation and inhibition in favour of the second one; indeed, despite cells intrinsic excitability is normal, the total excitatory synaptic drive decreases, while the total inhibitory drive increases. The incorrect balance between excitation and inhibition may cause deficits in learning and memory in RTT patients (Dani et al., 2005).

To further characterize the mechanisms underlying these alterations, the synaptic output was analysed (measuring the action-potential-evoked excitatory postsynaptic currents; EPSCs), resulting reduced in null neurons and enhanced in neurons over-expressing Mecp2, leading to the conclusion that Mecp2 regulates the magnitude of the synaptic output. The cause of this impairment was determined as a modification in the glutamatergic synapses number; indeed, the absence of Mecp2 leads to the loss of glutamatergic synapses, while its over-expression leads to an increment of them. This impairment was highlighted *in vitro* on glutamatergic neurons, but also *in vivo* on 2-weeks old mice, suggesting that Mecp2 has a fundamental role in the initial

formation of synaptic contacts during the early post-natal phases of neuronal development (Chao et al., 2007).

Further strengthening the role of Mecp2 in regulating synaptic plasticity, a mouse model presenting the truncation of the protein in position 308 shows impaired Long Term Potentiation (LTP) and Depression (LTD) mechanisms (Moretti et al., 2006), while a mouse model that over-express Mecp2 shows enhanced LTP (Collins et al., 2004).

To better characterize the role of Mecp2 in the brain and to evaluate the involvement of the different brain areas in the development of the phenotype, many mouse models lacking Mecp2 expression in precise brain districts were produced. For example, mice lacking Mecp2 selectively from the basolateral-amygdala (Adachi et al., 2009), the hypothalamus (Fyffe et al., 2008), dopaminergic and noradrenergic neurons (Samaco et al., 2009), or the GABA releasing neurons were generated (Chao et al., 2010). Interestingly, all these animals develop some aspects of RTT syndrome suggesting that RTT neurological traits are the sum of effects deriving from the malfunctioning of many, if not all, the different brain areas.

1.7 Is Mecp2 involved in pre-natal development or in neuronal maintenance?

Nowadays, it is still unclear since when the expression of Mecp2 is necessary for the correct neuronal functioning: whether starting from the first phases of neuronal development or whether exclusively for neuronal maintenance of mature cells.

Guy et al. investigated the role of Mecp2 in adulthood, through the reactivation of Mecp2 expression in *Mecp2* ko animals. Indeed, they generated a mouse line in which Mecp2 expression is silenced by a stop codon flanked by a loxP cassette, that can be removed by the expression of a ubiquitous Cre (bound to the estrogens receptor) activated by Tamoxifen (estrogens analogue) administration. The controlled injection of Tamoxifen to young adult mice (3-4 weeks old) induced the re-expression of, which mediated the elongation of survival and the global reduction of symptoms, therefore, demonstrating the reversibility of Mecp2-associated

symptoms, at least in mice (Guy et al., 2007). Nevertheless, this result did not clarify whether Mecp2 has a role in neuronal maintenance as adult neurons, lacking Mecp2 since early developmental phases, may not be properly mature (Guy et al., 2007).

To evaluate if the entity of the rescue induced by Mecp2 re-expression depends from the timing of re-expression, females harbouring a floxed stop cassette in the *Mecp2* gene were crossed with males expressing Cre under different promoters (Nestin, Tau and CamKIIα) that are activated in neurons at different developmental stages. Indeed, under the Nestin promoter, Cre starts to be expressed around E10 in neuronal and glial precursors; the Tau promoter is activated in post-mitotic neurons immediately after the exit from the cell cycle (still in the embryo), while the CamKIIα promoter is activated in neurons postnatally. Interestingly, when Mecp2 is re-expressed starting from early embryonic stages (Nestin and Tau promoters) the life span of the animals almost doubles compared to the ko. The rescue mediated by these promoters is almost undistinguishable, suggesting that RTT phenotype is mainly due to the lack of Mecp2 in neurons, rather than in glial cells. On the contrary, when re-activating Mecp2 expression in the post-natal brain (CamKIIα promoters) the increment of the life span is minimal (even if significant), further confirming that Mecp2 has a role in post-natal neurons, as the re-expression of Mecp2 in CamKIIα positive cells ameliorates the phenotype. However, the protein may exert important functions even during the pre-natal neuronal development, as the benefit obtained from the re-expression at earlier developmental time points is much more evident (Giacometti et al., 2007). This is in line with the previously reported study, in which mice lacking Mecp2 in Nestin positive cells since the first phases of neurogenesis (E10) developed stronger symptoms than the animals lacking it only from CamKIIα positive post-natal neurons (P1). Thus, the role of Mecp2 during pre-natal development is still debated, but its participation to these early phases is supported by growing evidences demonstrating that children and mice manifest subtle, but consistent, signs of the pathology in the so called "pre-symptomatic" stage, immediately after birth (Fehr et al., 2011; Nomura, 2005; Santos et al., 2006). Indeed, from retrospective analysis of children's first infancy, it was demonstrated that often RTT patients develop some behavioural abnormalities and muscle hypotonia before the overt onset of symptoms, together with a

disturbance of the sleep-wake rhythm. Moreover these children are often described as overly calm, or placid and have difficulties in rolling, crawling or pulling to stand (Fehr et al., 2011; Nomura, 2005). Analysing data deriving from 120 RTT affected girls, it was demonstrated that, at birth, they present a reduced occipito-frontal circumference, shorter length and lower weight (Huppke et al., 2003). Moreover, important evidences derive from RTT males that usually manifest strong signs of neurological dysfunction already at birth, suggesting that embryonic development could be somehow impaired when Mecp2 is absent (Smeets et al., 2012).

Alterations in the first post-natal period were also identified in mice, as ko animals display an increment of ultrasonic vocalizations (after separation from the mother) during the first week of life (compared to wt littermates) (Picker et al., 2006). Besides, another study highlighted that different parameters of maturation (as body weight and postural reflexes) are impaired in the pre-weaning period in null mice compared to wt littermates. Furthermore, the alteration of synapses, the reduced spontaneous activity and the diminished number of glutamatergic synapses previously described, appears early in post-natal development, during the "pre-symptomatic" phase (Chao et al., 2007; Dani et al., 2005). It would be of interest verifying whether these early alterations are ascribable to impairments of neurogenesis and/or maturation during the embryonic development. A first hint suggesting that Mecp2 may have a role since the first phases of generation and maturation of neurons derived from a study of Smrt et al., investigating adult neurogenesis in the dentate gyrus of the hippocampus. They demonstrated that this process, that resembles the embryonic cortical neurogenesis (starting from a Neural Precursor Cell (NPC), which asymmetrically divides, generating an immature neuron and another precursor), is altered when Mecp2 is absent, producing neurons that do not mature correctly (Smrt et al., 2007).

Through years, the few studies of the embryonic neuronal development in the RTT field focused mainly on cerebral cortex, probably because impairments in cortical morphology are one of the main features of RTT mice and patients (Armstrong, 2005; Fukuda et al., 2005). Moreover, RTT is characterized by strong cognitive deficits and, in some cases, by seizures that are mainly ascribable to cortical alterations (Smeets et al., 2012). Many groups agreed that Mecp2 expression in the

developing cerebral cortex follows neuronal maturation in mice, being detectable in early post-mitotic neurons reaching their final position in the cortex (Kishi & Macklis, 2004; Schmid et al., 2008; Shahbazian., 2002). Indeed, the protein was detected as early as at E12, but only in Cajal-Retzius (CR) cells, which are among the first neurons produced in the embryonic cortex. Later on in neurogenesis, Mecp2 is widely expressed by post-mitotic neurons residing in the entire Cortical Plate (CP). These works agreed on the lack of Mecp2 expression in progenitors populating the apical area of the developing cortex (Kishi & Macklis, 2004; Schmid et al., 2008; Shahbazian., 2002). Partially in contrast with this pattern of expression, it has been shown that a small proportion of progenitors (Nestin+) do express Mecp2 in rat embryonic cortex at E14 (Jung et al., 2003) and that it is expressed during all the phases of the central nervous system development in *Xenopus* and chicken embryos (Petazzi et al., 2014; Stancheva et al., 2003). More recently, *in vitro* experiments using neuroprogenitors (NPCs) and neural stem cells (NSCs) demonstrated that these cycling progenitors (Caballero., 2009; Okabe et al., 2010) and also adult NPCs *in vivo* (Li et al., 2014) do express Mecp2. All these evidences together did not produce a clear and definitive picture of whether and when Mecp2 is expressed during early corticogenesis. Thus, the real expression pattern of Mecp2 in the developing cortex should be better analysed; Mecp2 expression in cortical progenitors might suggest relevant functions even in the first phases of neurogenesis, when cells are still cycling.

1.8 Mecp2 and cellular proliferation

The role of MeCP2 in cell growth has been partially investigated in the past. The first evidence suggesting that MeCP2 expression influences cell proliferation dates back to 2002, when Balmer et al. performed single cell cloning of T lymphocytes, derived from heterozygous patients, segregating cells that correctly expressed the protein from the mutated ones. Surprisingly, they obtained a strongly reduced frequency of clones harbouring the *MECP2* mutation compared to the wt ones, concluding that although MeCP2 expression is not necessary for T lymphocytes survival, its absence is a disadvantage for cells growth (Balmer et al., 2002). Accordingly, the lack of

MeCP2 expression (obtained through the use of siRNAs directed towards *MECP2*) causes the reduction of cellular proliferation in different non-neuronal cell types, as glial cells (Nagai et al., 2005), prostate cells and cancer prostate cells (Bernard et al., 2006), NIH/3T3 (Babbio et al., 2012), MEF and MRC5 (Bergo et al., 2015). Moreover, *MECP2* repression has a positive effect on the dimension of cancer, reducing cell proliferation, invasion and migration in osteosarcomas (Meng et al., 2014).

The first suggestion of a possible mechanism through which MeCP2 could affect the cell cycle derives from the study of a post-translational modification of the protein. Indeed, the detection of the phosphorylation of Y120 led to the discovery of the association of MeCP2 to the centrosome and to the hypothesis that the protein could at least partly participate to cell cycle regulation through the interaction with this organelle (for example regulating the timing of mitosis, spindle geometry and microtubule nucleation). Accordingly, Bergo et al. demonstrated that the cell cycle progression of MRC5 resulted altered, when MeCP2 is absent, with the reduction of the proportion of cells in G1, and the increase of cells in G2/M. Moreover, MEF cells treated with siRNAs against Mecp2 are characterized by an increment of markers of mitosis (as pH3 and CyclinB1) and, accordingly, the duration of the mitosis of HeLa cells is prolonged (Bergo et al., 2015).

Given the central role of the brain in RTT pathogenesis, it would be of great interest linking the presented defects of cell growth to NPCs, as it is known that the neuronal phenotype strictly depends on its progenitorial stage (MuhChyi et al., 2013). Strengthening the importance of studying neural precursors, Li et al. demonstrated that, in adult NPCs, growth factors induce MeCP2 S421 phosphorylation, suggesting that Mecp2 PTM might be involved in cell proliferation. Indeed, a phospho-defective modification of S421 (S421A) causes reduced proliferation and an increased differentiation. Since no specific cell cycle phase was found affected, the authors suggest that this phenotype is related to the downregulation of the Notch signaling, which is normally downstream to this phosphorylation event. This work demonstrated, for the first time, that Mecp2 has a role in regulating the balance between proliferation and differentiation of adult NPCs; given the similarity of adult and embryonic neurogenesis it would be of interest to evaluate these mechanisms in

early development (Li et al., 2014). The first and so far unique study evaluating if Mecp2 expression affects embryonic neurogenesis, starting from cycling progenitors, was performed on chicken embryos. The over-expression (through *in utero* electroporation) of Mecp2 in the neural tube induces the reduction of the thickness of it after 48 hours, at a developmental stage (HH10) in which cells are mainly mitotically active progenitors. In agreement with an unbalance of proliferation and differentiation suggested by studies on aNPCs, Petazzi et al. highlighted a reduction of the number of cycling cells, possibly caused by a precocious differentiation; in fact, ectopically positioned neurons were identified. These results suggest that the over-expression of Mecp2 reduces the proliferative potential of progenitors and promotes differentiation, demonstrating for the first time that Mecp2 have a role in early development (Petazzi et al., 2014).

Overall it appears quite clear that a better investigation on the role of Mecp2 in NPCs is deserved, including an analysis on any possible effect on epigenetics. In fact, it is well-accepted that the methylation signature of a stem cell, together with other epigenetic modifications, determines its progressive cell differentiation, defining which cell type is going to mature from a single progenitor; it is easy to speculate that in the absence of Mecp2, the methylation of the DNA is not properly read and cells do not correctly mature (MacDonald and Roskams, 2009; MuhChyi et al., 2013).

2. MATERIALS and METHODS

2.1 Animals and tissues

The *Mecp2* mouse strain was originally purchased from Jackson Laboratories (003890 B6.129P2(C)-Mecp2<tm1.1Bird>/J). This strain, generated by Dr. Adrian Bird (Guy et al., 2001), is particularly poor in terms of progeny (heterozygous females are not too prone to feed and take care of pups, therefore are often replaced by foster mothers) and fairly difficult to maintain (ko male mice are sterile). Therefore, we bred heterozygous females with CD1 male mice, this backcrossing strategy was used until the 10th generation, after which the genetic background is usually considered clean enough for molecular testing. Ko mice on a clean CD1 background recapitulate the typical phenotype of the regular C57/BL6 original mice, with the advantage of having a larger progeny. Mice genotypes were analyzed through PCR on genomic DNA purified from tails. For the identification of the ko allele the forward primer was: 5'-ACCTAGCCTGCCTGTACTTT-3', while for the WT allele was: 5'-GACTGAAGTTACAGATGGTTGTG-3' and the reverse primer was 5'-CCACCCTCCAGTTTGGTTTA-3' (Miralvès et al., 2007). For the PCR amplification an incubation sequence of 30 seconds at 95°C, 30 seconds at 60°C and 40 seconds at 72°C was repeated for 35 cycles followed by a final incubation at 72°C for 7 minutes. The PCR product obtained was a single band of 450 base pairs (bp) for the ko, a single band of 400 bp for the wt and the two bands together for heterozygous animals.

Time pregnant females were generated by crossing overnight wt CD1 males with *Mecp2* heterozygous (–/+) CD1 females, the day of vaginal plug was considered E0.5. When needed, BrdU (30 mg/Kg) was intraperitoneally injected in time pregnant females at E14.5 and embryos were collected 24 hours later (E15.5). Tissues generated for microarray and RNA studies were dissected out of E15.5 embryos collected from anesthetized (Avertin, Sigma Aldrich) pregnant females, rapidly frozen on dry ice and stored at –80°C for future experiments. Embryos dedicated to histology were transcardially perfused with 5 ml 4% PFA under a

dissection microscope and post fixed in 4% PFA for additional 4 hours; cryoprotected overnight at 4°C in 30% sucrose, then embedded in Optimal Cutting Temperature (OCT) and kept at –80°C until sectioning. 12 μm sections were generated by slicing the tissues with a cryostat (Leica), sections were then stored at –20°C until further analysis.

All procedures were performed in accordance with the European Community Council Directive 86/609/EEC for care and use of experimental animals; all the protocols were approved by the Italian Minister for Scientific Research and by the local Animal Care Committee.

2.2 Histology, immunofluorescence and immunohistochemistry

As a general procedure, mounted, 12μm thick brain sections were washed in PBS and incubated for 1 hour in blocking solution (10% horse serum, 0.1% tritonX-100, PBS). In cases when antigen retrieval steps were necessary, samples were boiled in 0,01M Sodium Citrate pH 6,4, then washed in PBS, 10 min in 1% TritonX-100 (or PBS with Tween20 (1:1000) (PBST)) and 30 min in 0,1M Glycin. For the BrdU staining tissues were treated with HCl 1N at 37°C for 30 min, or with DNase (1 μg/ml, Roche, 11284932001) diluted in the blocking solution. Tissues were then incubated with the primary antibody diluted in blocking solution overnight at 4°C. Primary antibodies were used at the following concentrations: anti-Mecp2 (Sigma-Aldrich, 1:1000, rabbit), anti-Mecp2 (Cell signalling, 1:200, rabbit), anti-Nestin (Millipore, 1:200), anti-Tuj1 (Covance, 1:2000, mouse), anti-Reelin (DSHB, 1:20), anti-BrdU (Accurate, 1:1000, rat), anti-pH3 (Abcam, 1:3000, mouse), anti-RFP (Clontech, 1:500, rabbit), anti-GFP (Molecular Probe, 1:1000, chicken), anti-CyclinD1 (Neomarkers, 1:200, rabbit), anti-Tbr2 (eBioscence, 1:500, rat), anti-Pax6 (1:100, rabbit), anti-Ki67 (Vector, 1:100, rabbit), anti-Neun (Chemicon, 1:4000, mouse), anti-NeuroD1 (Santa Cruz, 1:50, goat). The day after, slices were washed for roughly 2 hours in PBS and, for the immunofluorescence, incubated for 1 hour with the secondary antibody (Alexafluor, Invitrogen, Molecular Probes) diluted (1:500) in blocking solution. Sections were then washed in PBS and incubated with DAPI (Invitrogen, 0,1 mg/ml in PBS) for 10 min, before mounting in Prolong Gold

(Invitrogen). For the immunohistochemistry, slices were incubated with the secondary biotinylated antibody (1:500, Vector Laboratory) diluted in blocking solution for 1 hour and treated with the Vector ABC kit (Vectastain) followed by staining with the Vector VIP peroxidase substrate kit. Sections were mounted with the DPX mountant.

Nikon microscope, objectives and software were used for the imaging of figure 1; Confocal Leica microscope, objectives and software was used for all the other figures. Panels were composed into figures using Adobe Photoshop.

For the measurement of the CyclinD1 intensity images were analyzed with ImageJ setting 3 different levels of threshold. Cells that resulted above the threshold were manually counted. An investigator blinded to the experiment counted all the cells.

2.3 Neurosphere generation

Timed-gestation female mice were anesthetized with Avertin to obtain E15.5 mouse embryos. The following procedure to generate Neural Progenitor Cells (NPCs) culture was first described by Gritti et al. and Magri et al. (Gritti et al., 1996; Magri et al., 2011). Briefly, embryos were individually dissected in PBS and the neocortex was transferred in Dulbecco's Modified Eagle Medium/F12 (DMEM/F12) and dissociated by extensive enzymatic digestion with Papaine (Sigma) and DNase (0,1 mg/ml, Roche, 11284932001), followed by mechanical trituration (using a pipette) till no residual tissue was anymore visible. Cells were then grown in complete medium (DMEM/F12, 30% Glucose, 7,5% $NaHCO_3$, 1% L-Glutamine, 1% Pen/Strep, 2mg/ml Heparin, Hormone Mix, 20 ng/ml Epidermal Growth Factor (EGF), 10 ng/ml basic Fibroblast Growth Factor (bFGF)), in which cells spontaneously form neurospheres (Gritti et al., 1996). Neurospheres were then dissociated in single cell cultures, plated on matrigel-coated coverslips and fixed with 4% PFA to produce samples to be analyzed by immunofluorescence experiments.

For the neurosphere generation assay 100 singles cells were re-suspended in 100 µl of complete medium in a well of a 96-well plate. After 3 days the generation of spheres was evaluated.

2.4 Time-lapse imaging

The time-lapse imaging experiments were performed with a Zeiss Axiovert S100 TV2 microscope. Cells were plated and maintained in complete medium for 48 hours at 37°C with 5% CO2, images were taken every 10 minutes. Single cells were then analysed for the mitosis duration.

2.5 RNA purification, cDNA synthesis and quantitative PCR

The RNA was extracted either from neurospheres or embryonic cortexes; tissues were homogenized in RLT buffer (RNeasy Lysing Buffer from the RNeasy Mini kit, Qiagen) using a glass-glass potter, while cells were vigorously resuspended in the same buffer; the whole volume was loaded on a RNAeasy mini column (Qiagen), following the kit protocol. The RNA was eluted RNA/DNAse free H_2O.
The cDNA synthesis was performed using a QIAGEN kit (RT^2 First Strand Kit). The qPCR for the intracellular signaling factors (Fig. 10C) was performed using SYBR Green (Life Technology) as a fluorescent dye. With the following primers:

Gene		Sequence (5' -> 3')	Tm
18S	ff	GTAACCCGTTGAACCCCATT	61.8
	rev	CCATCCAATCGGTAGTAGCG	61.6
Adcy9	ff	CAACAGCGTGAGGGTCAAGAT	62.3
	rev	CATGGAGTCGAATTTGGGGTC	60.7
Camk2b	ff	GCACGTCATTGGCGAGGAT	62.7
	rev	ACGGGTCTCTTCGGACTGG	62.9
Camk2d	ff	CTAGGGACCATCAGAAACTGGA	60.6
	rev	GGATCTGCTGAATGCAATGACTG	61.8
Gnaq	ff	GGTCGGGCTACTCTGACGA	62.7
	rev	ACTTGTATGGGATCTTGAGCGT	61.2
Mecp2	ff	AAACCACCTAAGAAGCCCAAAT	60.7
	rev	TTGACAAACAAGTTTCCCAGGG	60.4
Plcg2	ff	GTGGACACCCTTCCAGAATATG	60.1
	rev	ACCTGCCGAGTCTCCATGAT	62.6

The qPCR for the glutamatergic receptors and the ionic channels (Fig. 10A, B) was performed using the Synaptic Plasticity RT^2 Profiler PCR Array and the Neuronal Ion Channels RT^2 Profiler PCR Array (Qiagen) respectively.

2.6 Microarray

The quality of the RNA extracted from 3 wt and 3 ko E15.5 cortices was assessed with the Agilent Bioanalyzer microfluidics-based system (Agilent technologies), samples with RNA integrity number lower than 8 were discarded. The gene expression of whole transcriptome was performed with the Illumina Direct Hybridization Assays according to the standard protocol (Illumina Inc.). For each sample 500 ng of total RNA were reverse transcribed according to the Illumina TotalPrep RNA Amplification kit (Ambion) and cDNA was generated. Washing, staining and hybridization were performed according to the standard Illumina protocol; each sample was hybridized onto Illumina MouseRef-8 Expression BeadChip arrays. Hybridization and scanning were performed according to the manufacturer's indications on an Illumina iScan System and data were processed with GenomeStudio; array data were processed with quantile normalization in Genome Studio software before further analysis, average signals were calculated on gene-level data according to the standard Illumina detection cutoff (detection p-value lower than 0.001).

2.7 Gene Set Enrichment Analysis

At first the GSEA software organizes the genes of the dataset in a ranked list based on their expression value, then it determines the distribution of a set of genes (gene set) throughout the ranked gene list. To obtain a numerical measurement of the representation of a gene set in one experimental group or the other the GSEA software calculates an Enrichment Score (ES). The ES increases when a gene belonging to a gene set is found in the ranked list, the degree of the increment depends on the amplitude of the differential expression. Then the software

determines the significance of the ES, by an empirical gene set-based permutation test procedure. The gene sets can be custom-made or can be downloaded from the Moleculare Signature Database (www.broadinstitute.com).

2.8 Neuronal cultures

Cortexes from E15.5 animals were dissected out and digested with Trypsin 0,25% and mechanically dissociated by pipetting. Single cells were plated at low density (30.000 cel/well in 6-multiwell plate) on glass coversplip coated with poly-L-lysine (0,1 mg/ml). Neurons were grown in culture medium: Neurobasal, B27 (1:50), Glutamax 1% and Pen-Strep 1%.

2.9 Calcium imaging

Cortical neurons (from 3 ko and 4 wt animals) were loaded with calcium-sensitive dye (10 mM Oregon Green 488 BAPTA-1 AM, Molecular Probes) in Neurobasal medium for 1 hour at 37°C. The detection of calcium responses was performed in KRH (Krebs'-Ringer's-Hepes: 125 µM NaCl, 5 µM KCl, 1,2 µM $MgSO_4$, 1,2 µM KH_2PO_4, 15 µM Hepes, 6 µM Glucose, 2 µM $CaCl_2$ at pH 7,4). The stimulation with 25 µM glutamate was performed in the presence of Nifedipine (20 µM), Tetradotoxin (1 µM), and Bicuculline (20 µM) through a perfusion system (Warner Instruments, Hamden, CT, USA). The electrical field stimulation was performed in the presence of CNQX (20 µM, 6-cyano-7-nitroquinoxaline-2,3-dione), APV (50 µM, R-2-amino-5-phosphonopentanoate) and Bicuculline (20 µM) using a stimulation chamber (Warner Instrument). Calcium transients were induced with a stimulus train of 2 seconds (duration 1 ms; amplitude 90 mA) at 30 Hz (Pozzi et al., 2013) using a train generation unit (Digitimer Ltd, DG2A) connectd to a stimulus isolation unit (SIU-102; Warner Instruments). Recording chambers were placed on the stage of an IX-71 inverted microscope (Olympus) equipped with an EMCDD (electron-multiplying CCD) camera (Quantem 512x512, Photometrics). The illumination was obtained using a light-emitting diode single LED (Cairn Research

Optoled; 470 nm) and a related GFP filter; Regions of interest (ROIs) of about 15-pixel diameter (corresponding to ≈12 μm) were drawn on the cell cytoplasm of virtually all the cells in the recorded field. Time-lapse recording of calcium dynamics was performed with an acquisition rate of 5 Hz for 600 s and off-line analysed with the MetaFluor software (Molecular Devices). Calcium responses were measured as ΔF (Fmax–F0) with respect to the baseline (F0). A total of 10–15 neurons were analysed for each field. Cumulative data were then analysed through Kolmogorov–Smirnov statistic to verify non-parametric distribution.

2.10 Morphological analysis

Cortical neurons from 3 ko and 4 wt animals were prepared as previously described and morphological analyses were performed at DIV3. Dendrites were highlighted through the use of the anti-Tuj1 antibody, while the nuclei through the use of DAPI. Neurites length was measured only on single neurons, not in contact with other neurons, using the NeuronJ plugin of ImageJ. Neurites were considered as the Tuj1 positive structure starting from the soma. They were classified as long when their length was above 100 μm, or short when shorter than 100 μm. The size of the nuclei was measured on the DAPI staining using ImageJ.

3. RESULTS

Although RTT is considered a neurodevelopment pathology, the function of Mecp2 has been thoroughly investigated only during late phases of development, possibly because children were considered normal at birth. Nowadays, evidences of subtle but consistent symptoms in the so-called "pre-symptomatic" phase are growing suggesting possible roles of Mecp2 during early phases of development (Chao et al., 2007; Fehr et al., 2011; Nomura, 2005; Santos et al., 2007). For this reason, and given the cortical phenotype affecting both RTT patients and animal models, we started investigating the role of Mecp2 during corticogenesis.

First, we searched for previous evidences of Mecp2 expression in the cortex. Different works agreed that Mecp2 expression follows neuronal maturation in mice, as it was detected only in early post-mitotic neurons reaching their final position in the cortex and not in cycling progenitors (Kishi & Macklis, 2004; Schmid et al., 2008; Shahbazian., 2002). However, these evidences have been challenged by the recent detection of Mecp2 expression in *in vitro* cultivated cycling cells such as NSCs and induced pluripotent cells (IPCs) (Caballero., 2009; Okabe et al., 2010). Taken together, these data do not produce a clear and definitive picture of whether and when Mecp2 is expressed during early corticogenesis or whether its expression is confined to mature neurons only.

To map Mecp2 expression during cortical development, we stained embryonic tissues (using null animals as negative controls) beginning from embryonic day 10.5 (E10.5, Fig. 3.1a, a'), a time point at which the cortical wall is populated by neuroepithelial cells, that, later on, will give rise to the different types of cortical NPCs (apical and basal). Such progenitors will, eventually, give birth to the glutamatergic neurons populating the cortex (Florio and Huttner, 2014). Stunningly, at such time point we were able to detect low but consistent levels of Mecp2, that continues to be expressed throughout embryonic cortical development (Fig. 3.1a-c) by all cell types: progenitors (Nestin+; Fig. 3.1d), immature/maturing neurons (Tuj1+; Fig. 3.1e) and Cajal Retzius cells (CR; Reelin+; Fig. 3.1f). From these analyses it is clearly visible that Mecp2 levels are indeed lower in the NPCs

compared to mature neurons (Fig. 3.1b), possibly explaining why it was hardly detected in this population in the past.

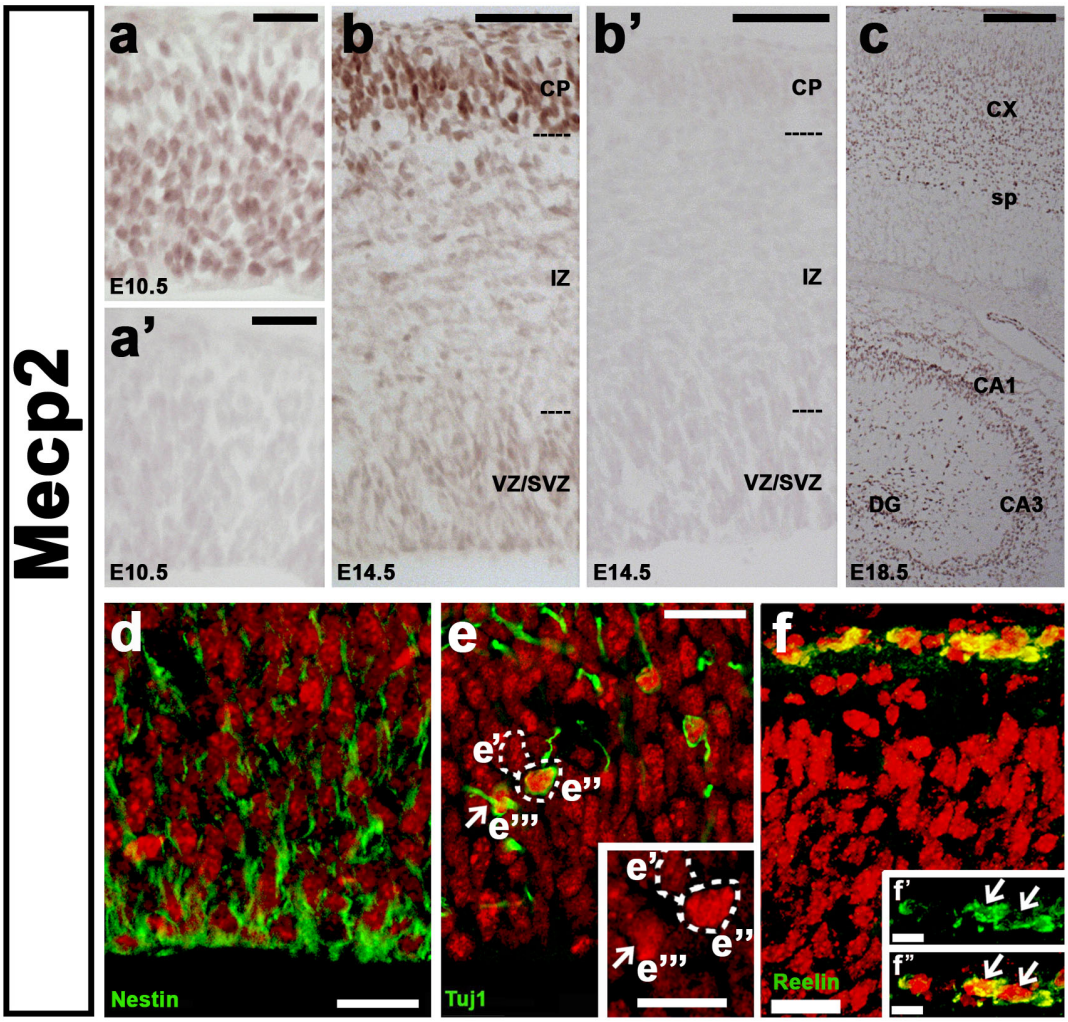

Figure 3.1. Mecp2 is expressed by all the cell types composing the developing embryonic cerebral cortex. Mecp2 is detectable, at least, starting from E10.5 (**a**) and continues to be expressed during all the cortical development (**b:** E14.5; **c:** E18.5), throughout the entire cortical thickness. Ko tissues were used as negative controls (**a', b'**). All the different cell types expressing Mecp2 were identified through specific markers: Nestin to recognize progenitors (**d**), Tuj1 for neurons (**e**) and Reelin for CR cells (**f**). Scale bar: 25 µm in a, a', d, e, e', f. 50 µm in b, b'. 100 µm in c. 10 µm in f', f'' (Bedogni et al., 2015).

The discovery that Mecp2 is expressed in the cortex from the earliest phases of neurogenesis raised the question of whether and what functions it might play in these populations. Given the well-accepted role of Mecp2 as transcriptional regulator, we performed a microarray analysis on E15.5 ko cortices compared to wt controls, to evaluate the transcriptional perturbations generated by lack of Mecp2. The choice of

focusing on E15.5 has different advantages: (i) it corresponds to the peak of neurogenesis in mice; indeed, at this time point the cortex is populated by a mixture of progenitors, maturing and mature neurons; (ii) E15.5 is a stage at which cells are mainly glutamatergic, without glial cells or massive infiltration of GABAergic interneurons possibly masking effects driven by the heterogeneity of the different cellular populations.

A first analysis of the transcriptional profile through regular bioanalytical approaches did not highlight any particularly strong effect: imposing a fold change cut-off of 1.3, we only obtained 270 deregulated genes, that became 11 with a fold change of 2 and 0 with higher fold changes. Furthermore, applying a p-value threshold of 0.05, without fold change cut-offs, only 135 genes resulted differentially expressed (Fig. 3. 2A). Moreover, the standard gene ontology (GO) analysis did not reveal any solid molecular or functional relationships between the modulated genes (Bedogni et al., 2015). Such results, besides not being particularly informative, are anyway in line with the fact that Mecp2, globally binding to methylated sequences, modestly and regulates the transcription of the vast majority of genes (Skene et al., 2010). In line with this, it is thus not possible to detect strong transcriptional deregulations of single genes. Given such a small variation in amplitude, we hypothesized that analysing groups of (possibly mildly) deregulated genes rather than single transcripts could be more informative. The rationale is that the sum of even mild deregulations affecting a statistically significant number of genes of the same group could highlight biological effects otherwise masked. The Gene Set Enrichment Analysis (GSEA) provides this possibility. The GSEA software considers the entire dataset obtained from a microarray analysis without imposing any threshold, as it considers each transcriptional variation, even if small in amplitude, that normally would be considered “below threshold”. The GSEA focuses on groups of selected genes (gene sets) gathered because of common features, such as being part of the same biological pathway, and imposes these gene sets on the dataset (previously ordered based on expression levels) determining whether they are enriched in one of the two conditions (in our case *Mecp2* ko or wt; Subramanian et al., 2005).

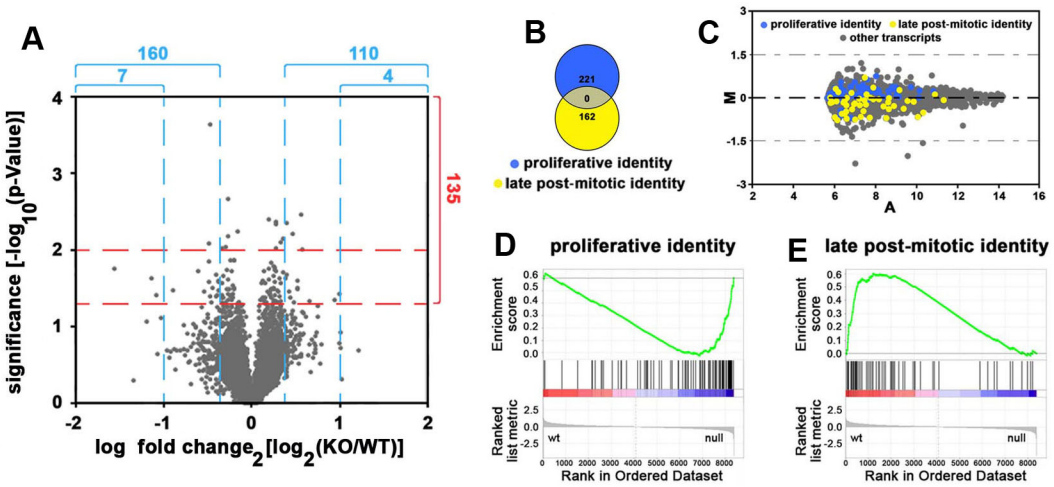

Figure 3.2. E15.5 *Mecp2* ko cortices display a mildly deregulated expression profile. Data obtained by the microarray analysis of E15.5 cortices were graphed on a volcano plot (**A**), displaying the statistical significance versus the differential expression of genes (fold change). Blue lines represent 1.3 and 2 fold change cut offs; red lines correspond to 0.05 and 0.01 p-values. The microarray was analysed using the GSEA with two non-overlapping custom-made gene sets (**B**), representing the proliferative and the post-mitotic compartment of the cortex. The two gene sets segregate in either the wt or ko samples as highlighted by the MA plot (**C**; M: $\log_2$(KO/WT); A: 1/2$\log_2$(KOxWT)) and by the GSEA output (**D, E**) (Bedogni et al., 2015).

At first, we exploited the GSEA software through the creation of two custom-made gene sets, composed by the genes sharing the common feature of being expressed by cells located in different subareas of the developing cortex. This was made possible thanks to the data produced by Ayoub et al. (2011); in their work, in fact, the authors determined the gene expression profile of different mouse cortical areas (VZ/SVZ, IZ, CP) dissected through laser capture on E14.5 cortexes. In order to clearly segregate genes describing either the VZ/SVZ population (cycling progenitors with a proliferating identity) or the CP population (maturing/mature neurons with a late post-mitotic identity) we applied a fold change cut-off of 20 on the data (Fig. 3.2B). At first, by superimposing these two sets of genes on a plot representing the amplitude of expression of each gene (A) related to its fold change (M) (MA plot), we highlighted a peculiarity in their segregation, as in ko samples the group defining the proliferative identity was for the most part up-regulated, while the group defining the post mitotic/mature identity was down-regulated (Fig. 3.2C). The GSEA confirmed the statistically significance of the enrichment of the proliferation identity in the ko samples and the late post-mitotic identity in the wt samples (Fig. 3.2D). We interpreted these results hypothesizing that *Mecp2* null cells might proceed through

different phases of neurogenesis at a lower rate, therefore delaying the maturation process in the ko cortex. Furthermore, to elucidate what molecular mechanisms could be deregulated when Mecp2 is absent, we superimposed all the curated gene sets, representing canonical pathways collected in the Molecular Signature Database (MSigDB, version 3.1; www.broadinstitute.org) on our dataset. Figure 3.3 summarises the pathways most significantly enriched in the ko (A) and wt (B) condition; such pathways were selected according to their statistical significance ($p<0,00001$) and their involvement in mechanisms of cortical development.

A

Gene Sets	SIZE	ES	NOM p-val
BIOCARTA_G2_PATHWAY	16	-0.67	0.0
REACTOME_M_G1_TRANSITION	53	-0.54	0.0
REACTOME_MITOTIC_PROMETAPHASE	54	-0.54	0.0
REACTOME_MITOTIC_M_M_G1_PHASES	109	-0.53	0.0
REACTOME_S_PHASE	71	-0.50	0.0
REACTOME_G1_S_TRANSITION	68	-0.50	0.0
REACTOME_CELL_CYCLE	229	-0.49	0.0
REACTOME_CELL_CYCLE_CHECKPOINTS	72	-0.49	0.0
KEGG_CELL_CYCLE	73	-0.47	0.0
REACTOME_MITOTIC_G1_G1_S_PHASES	81	-0.46	0.0
REACTOME_CELL_CYCLE_MITOTIC	191	-0.45	0.0

cell cycle cell cycle phases

B

Gene Sets	SIZE	ES	NOM p-val
REACTOME_TRAFFICKING_OF_AMPA_RECEPTORS	18	0.68	0.0
REACTOME_ION_CHANNEL_TRANPORT	21	0.66	0.0
REACTOME_PHOSPHOLIPASE_C_MEDIATED_CASCADE	22	0.66	0.0
REACTOME_POTASSIUM_CHANNELS	30	0.60	0.0
REACTOME_G_ALPHA_I_SIGNALING_EVENTS	47	0.53	0.0
REACTOME_PLC_BETA_MEDIATED_EVENTS	21	0.64	0.0
REACTOME_CA_DEPENDENT_EVENTS	16	0.66	0.0
REATOME_UNBLOCKING_OF_NMDA_RECEPTOR_GLUTAMATE_BINDING_AND_ACTIVATION	7	0.83	0.0
REACTOME_SIGNALING_BY_GPCR	178	0.38	0.0
KEGG_CALCIUM_SIGNALING_PATHWAY	61	0.53	0.0
KEGG_PHOPHATIDYLINOSITOL_SIGNALING_SYSTEM	43	0.54	0.0
REACTOME_RAS_ACTIVATION_UPON_CA2_INFLUX_THROUGH_NMDA_RECEPTOR	7	0.81	0.0
REACTOME_DAG_AND_IP3_SIGNALING	17	0.67	0.0
REACTOME_VOLTAGE_GATED_POTASSIUM_CHANNELS	13	0.70	0.0

ionic channels intracellular effectors glutamatergic receptors

Figure 3.3. GSEA analyses performed using canonical pathways as gene sets gave the following outputs: (**A**) the canonical pathways enriched in the ko condition are mainly related to the cell cycle in general (in red) or to cell cycle phases (in blue); while, the pathways enriched in the wt condition (**B**) are related to three main categories: ionic channels (in red), intracellular effectors (in blue) and glutamatergic receptors (in green).

Interestingly, the pathways enriched in the ko samples are related to cell cycle and the progression through its phases, while those enriched in the wt condition can be divided in three different groups according to their involvement with glutamatergic receptors transmission (in green), ionic channels transmission (in red) and intracellular effectors (in blue). All these processes are obviously crucial for defining neuronal functionality. These findings are in agreement with the maturation defect previously hypothesized: ko cells, compared to wt, seem to be hold in a progenitorial

condition (VZ/SVZ features) and express reduced levels of genes that are characteristic of maturing/mature neurons (CP features).

To validate the obtained data and assess whether the maturation defect begins from the earliest processes of neurogenesis, we made use of *in vitro* cultured Neural Progenitor Cells (NPCs) generated from E15.5 cortices. Studying progenitors is of particular interest when evaluating neurogenesis, as the fate of a newly born neuron depends on both its genetic program and its ability to receive external stimuli since its progenitor stage. Accordingly, the neuronal fate strictly depends on the proper progression through the cell cycle (Arai and Pierani, 2014).

At first, we confirmed the *in vivo* data (Fig. 3.1d), demonstrating that wt NPCs express the mRNA of Mecp2 and produce the protein (Fig. 3.4A, b). Subsequently, given that NPCs, maintained in proliferating conditions, spontaneously form neurospheres, we analysed the number of neurospheres generated starting from single cells in a short time lapse as a first evaluation of cell cycle progression. We obtained NPCs from wt and ko animals and discovered a slight but statistically significant reduction in the number of spheres produced in the ko condition compared to wt controls (Fig. 3.4C). Further, we measured the bromodeoxyuridine (BrdU) uptake of NPCs cultivated in adhesion, derived from *Mecp2* heterozygote neurospheres (figure 3.4D); in which wt and ko cells grow in a roughly 50% proportion, in order to constrain as much as possible any variability generated from differences in cell culture density. BrdU (10 μM) was administered to NPCs every 2 hours and a fraction of cells was collected 30 min after each administration. A total time frame of 14.5 hours, shorter than an entire cell cycle, was selected in order to avoid that the same cell could incorporate BrdU twice. The cumulative BrdU incorporation analysis highlighted a reduction in the proportion of ko cells uptaking BrdU (Fig. 3.4D) and, moreover, the ko reached saturation later than the wt (6,5h wt vs 8,5 ko; Fig. 3.4d'). Thus, in agreement with our first data and with previous publications the lack of Mecp2 reduces the proliferation rate of cycling cells (Babbio et al., 2012; Bergo et al., 2015).

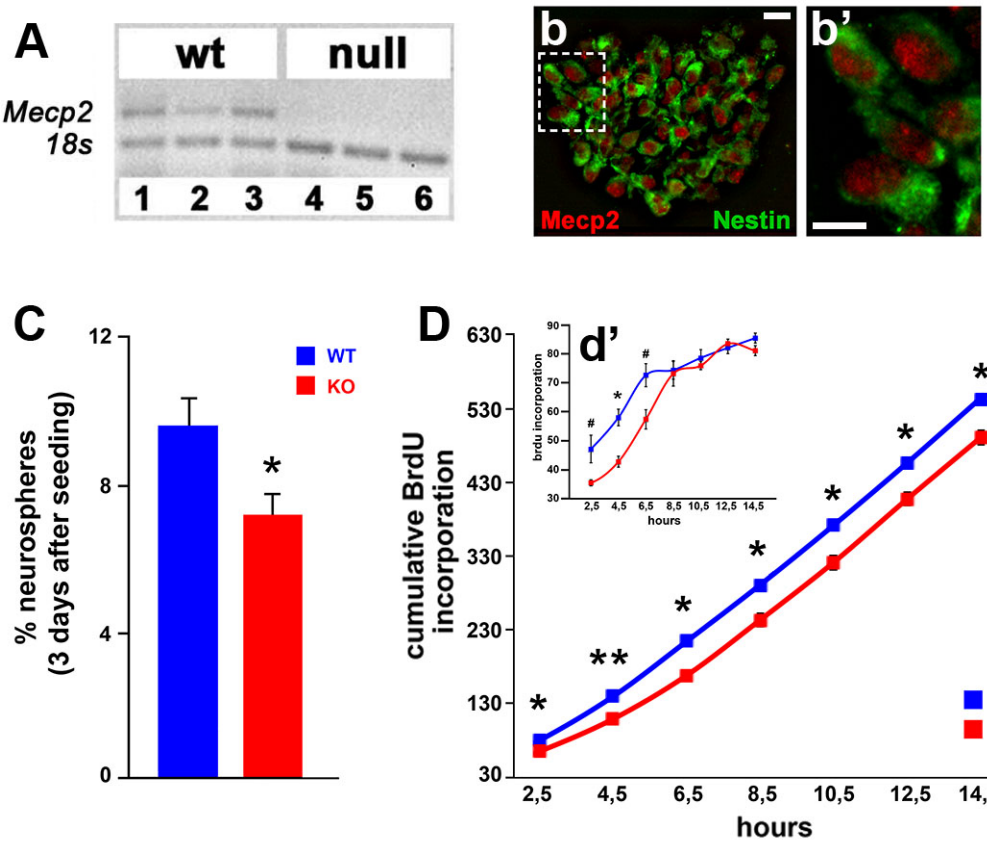

Figure 3.4. Mecp2 is expressed in NPCs and its absence alters the entrance in S phase. Confirming *in vivo* data, wt NPCs *in vitro* express the mRNA of Mecp2 (**A**) and produce the protein (**b, b'**). The lack of Mecp2 from NPCs causes the reduction of the percentage of neurospheres formed after 3 days of culture in cycling conditions (**C**). Moreover, ko NPCs incorporate less BrdU compared to wt samples, indicating that the lack of Mecp2 alters the initiation, or the progression, in S phase (**D:** cumulative analysis of BrdU incorporation; **d':** BrdU incorporation). Scale bar: 20 µm in b. 10 µm in b'. Values represent the mean ± the SEM (n=3 wt and ko for the neurosphere generation assay; n=3 heterozygous animals for the BrdU incorporation). Statistical analysis: Student's t test *= p<0.05; **=p<0.01; #= almost 0.05. Kolmogorov–Smirnov testing revealed a p <0.001 for the cumulative analysis in **D.**

We proceeded addressing which phase of the cell cycle affects the progression of ko progenitors. We started administrating BrdU to cycling NPCs *in vitro* (again from heterozygous embryos) and collecting the cells after 30 minutes; this experiment, lasting only half an hour was specifically designed to study, at a roughly steady state, the percentage of cells entering the S phase. In line with the data described in figure 3.4D, we highlighted a reduction in the proportion of ko cells entering in the S phase (Fig. 3.5A, B). To count the percentage of cells in the G2 phase we exploited the well-recognized dotted staining of phospho-histone H3 (pH3) (Van Hooser., 1998), counting the number of dotted pH3 stained nuclei that incorporated BrdU one or two

hours before collection (Fig. 3.5C). At both these time points we did not obtain any effect driven by the lack of Mecp2 (Fig. 3.5D). Next, we measured the M phase length through a time lapse imaging approach. Considering that a complete M phase lasts from when cells appear rounded in shape to the loss of any contact between the two daughter cells (Fig. 3.5E), we did not detect any defect in the duration of M phase (Fig. 3.5F).

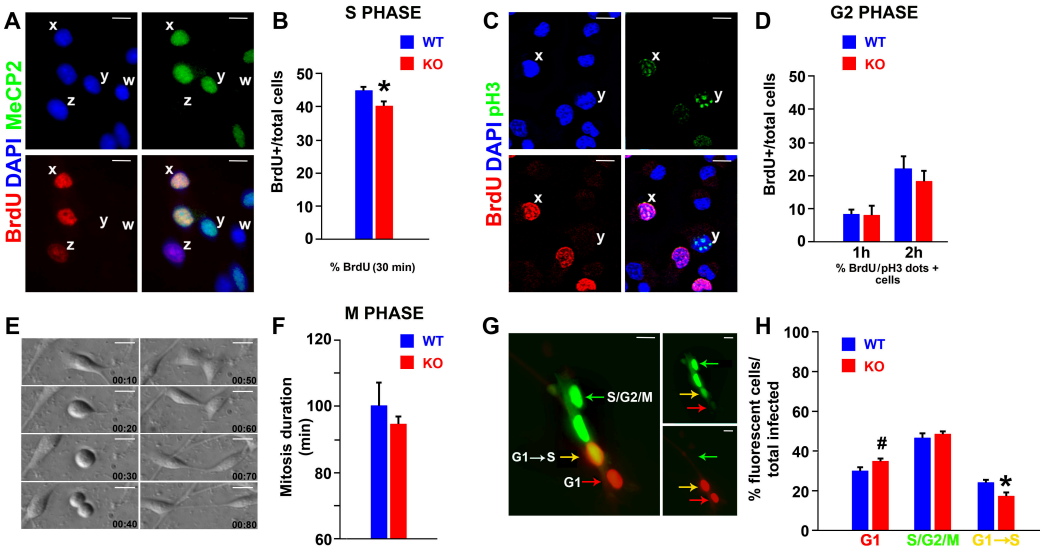

Figure 3.5. Lack of Mecp2 impairs cell cycle progression. Cultures of NPCs generated from heterozygous embryos (**A**) display cells expressing Mecp2 (**x, y**) and cells not expressing Mecp2 (**w, z**) that may or may not have incorporated BrdU (**x, z** and **y, w**). The proportion of ko NPCs that incorporated BrdU for 30 minutes (thus in S phase) is reduced (**B**), while no changes (**C**, **D**) were detected in the proportion of $BrdU^+$ wt and ko cells that, displaying a dotted pH3 staining, are considered in G2 (**x, y**). The length of the M phase (measured through time lapse imaging; **E**) is not impaired (**F**). Using Fucci probes, it was possible to demonstrate that there is an accumulation of cells in the G1 phase, that corresponds to a reduction of cells passing from the G1 to the S phase (**H**). Green cells are in the S/G2/M phases, red cells are in the G1 phase while yellow cells are proceeding from the G1 to the S (**G**). Scale bar: 10 µm for all the images. Values represent the mean ± the SEM (n=3 heterozygous animals for the BrdU incorporation, roughly 500 cells per time point, per animal were counted; n=3 ko and wt for the time lapse, roughly 400 cells per animal were analysed; n=3 ko and wt, roughly 500 cells per animal were counted for Fucci). Statistical analysis: Student's t test *= $p<0.05$; #= almost 0.05.

To summarize, the observed lack of defects in the G2 and M phases (Fig. 3.5D, F) together with the delay in the S phase entrance (Fig. 3.5B) suggest that *Mecp2* null NPCs defects in cell cycle progression might be due to a prolonged G1 phase. We, thus, proceeded evaluating the length of the G1 phase through the use of Fucci

probes (Fluorescent Ubiquitination-based Cell-Cycle Indicator) that are specifically designed to be expressed in different cell cycle moments (Sakaue-Sawano et al., 2008). Cells expressing these probes result red in colour when in G1, green during the S/G2/M phases and yellow when progressing from the G1 to the S phase (Fig. 3.5g). This approach permits to analyse all the cell cycle phases simultaneously on the same cells. In line with the results previously described, we did not detect any difference in the proportion of cells in S/G2/M phases (green); we found an increase in the number of cells in the G1 phase (red), and, importantly, a reduction of the percentage of yellow ko NPCs (G1 to S progression) compared to wt (Fig. 3.5H). To be noticed and in accordance with the absence of any gross morphological defect in ko cortices and the roughly correct layering of the cerebral cortex of both RTT animal models and patients, the impairments highlighted so far are not massive (Kishi and Macklis, 2004). Recently, we published a study demonstrating, *in vitro*, a reduction of cells in G1 and the increment of cells in G2/M; however such effect was highlighted using a different cell type (MRC-5; fetal lung fibroblast). Further, lack of Mecp2 in MRC-5 causes a strong affection of cell cycle (less than $50x10^4$ cells after 30h of cultures compared to more that $200x10^4$ for the wt; (Bergo et al., 2015). This result is by no means comparable to the slight reduction of neurosphere formation.

Our *in vitro* approach was designed to simplify the analysis of cell cycle progression. However, we are well aware that the behaviour of NPCs cultivated *in vitro* can't be compared to the complexity of *in vivo* conditions; indeed, *in vitro* NPCs continuously cycle without differentiating in neurons and do not receive external cues from post-mitotic neurons.

To obtain *in vivo* data, we stained E15.5 cortices for CyclinD1, the principal marker of the G1 phase. As expected, we highlighted an increase in the proportion of CyclinD1 positive cells in *Mecp2* null cortices with respect to wt (Fig. 3.6A). To further characterize this increment, we classified the CyclinD1 positive cells in three different groups according to the signal intensity: high or low (defining either full G1 phase (high) or entering/leaving it (low)) and intermediate (defining cells in between these two sub-phases; Fig. 3.6b, c). Interestingly, the increased CyclinD1 levels are ascribable to an increment in the proportion of weakly positive cells, thus either just

entered in the G1 phase or ready to leave it, in agreement with the reduction in cells passing through G1 to S highlighted by the Fucci approach (Fig. 3.5H).

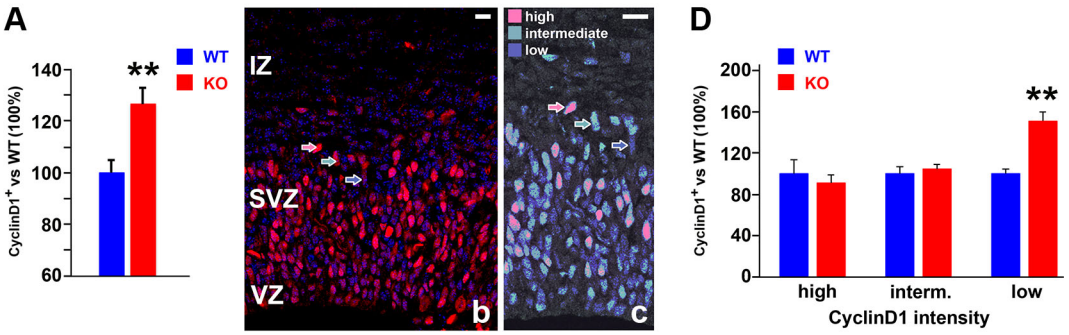

Figure 3.6. E15.5 cortices display an increment of the G1 marker CyclinD1. The percentage of CyclinD1 positive cells is increased in ko cortices (**A**). CyclinD1 expression displays three different levels of intensity as in **b**, where a typical staining in wt tissues is represented. The intensity of the staining corresponds to the three different pseudo colours depicted in **c**: low (blue cells), intermediate (green), high (pink). The increment in the percentage of CyclinD1 cells is mainly due to cells expressing it at a low intensity (**D**). Cells in a fixed grid were counted (an average of 500 cells per animal). Scale bar: 30 µm. Values are expressed as percentage versus wt ± the SEM (n=9 wt and 6 ko). Statistical analysis: Student's t test *= $p<0.05$; **=$p<0.01$.

Neurogenesis in the cerebral cortex is characterized by the expression of several markers expressed in a tightly regulated timing (Hevner et al., 2006). Even within the progenitor stage it is possible to find specific features differently marking two main classes of cortical progenitors: apical and basal recognizable according to the expression of Pax6 and Tbr2 respectively. Given the defects highlighted by the bioinformatics approach and our *in vitro* and *in vivo* evidences, we focused on the establishment of the identity of both these two classes of cortical progenitors. Interestingly, the E15.5 *Mecp2* null cortices display a slight, but statistically significant, increase in the number of Pax6 positive cells and a stronger, in magnitude, increase in the number of Tbr2 positive cells (Fig. 3.7B). Interestingly, the increment of these two markers accompanies the increase in the number of double positive cells (Fig. 3.7B). This evidence is even more important considering that by BrdU injection (24 hours before tissues collection) we detected a strong increase in the number of triple positive cells (Pax6/Tbr2/BrdU) in ko samples compared to wt (Fig. 3.7D). Given that BrdU enables to roughly synchronize the cells that are in the S phase at the time of the injection, these data suggest that ko

basal progenitors (Tbr2 positive) retain part of the identity of apical progenitors (Pax6 positive), a phenotype that physiologically should characterize only a reduced number of transitioning cells.

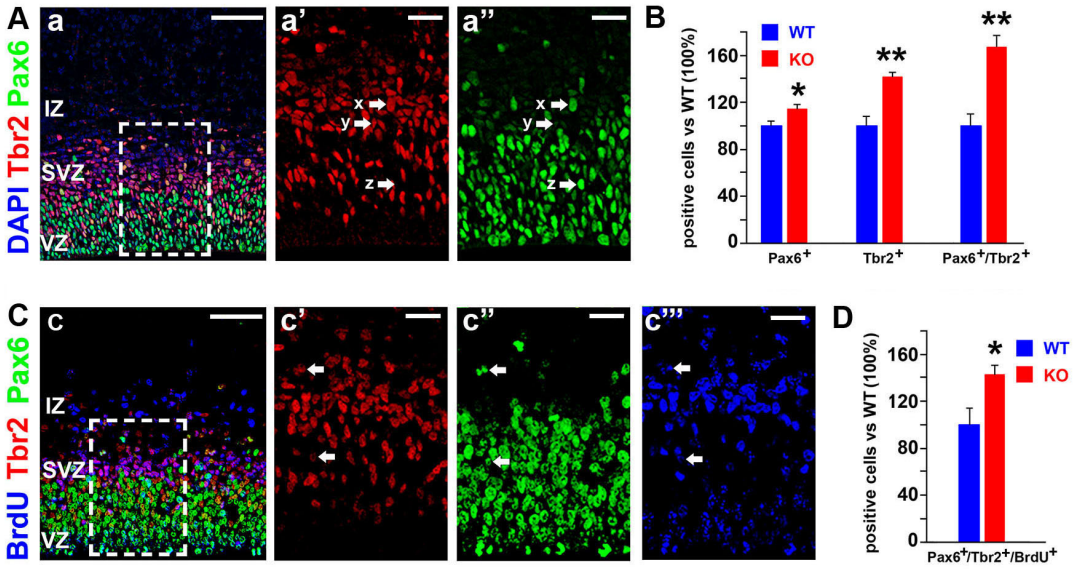

Figure 3.7. Lack of Mecp2 alters the expression of different progenitorial markers. A: E15.5 ko cortices display an increased number of Pax6 (marker of apical progenitors; **a''**) cells, Tbr2 (marker of basal progenitors; **a'**) cells and double positive cells (cells transitioning between the two conditions) (**B**). **C:** the increment is confirmed also when cells are synchronized using BrdU injection (24 hours before the tissue collection; **D**). The images displayed in **A** and **C** represent the expression of the different markers in wt animals. Roughly 500 cells (counted in a binned grid) were counted per animal according to Pax6 or Tbr2 immunoreactivity. Scale bar: 50 µm. Values are expressed as percentage versus wt ± the SEM (n=9 wt and 6 ko). Statistical analysis: Student's t test *= $p<0.05$; **=$p<0.01$.

A defect in the transition between the stage of progenitor to the post-mitotic one could thus be hypothesized. We proceeded testing the switch between these two cell populations by administering BrdU to pregnant mice and collecting embryos after 24 hours (E14.5 on E15.5), a time window sufficient for a complete cell cycle, enabling the cell to either re-enter in the cycle or differentiate. By using a marker of cycling cells such as Ki67, we detected in the *Mecp2* null cortices a slight increase in the number of Ki67/BrdU cells (Fig. 3.8B). As we did not expect cells to re-enter the cell cycle faster in the ko animals compared to wt, we hypothesized that the observed alteration could be caused by a slower transition of *Mecp2* defective cells from the proliferative to the post-mitotic state, therefore leading to a less sharply defined cell

identity. To confirm this hypothesis, we stained E15.5 cortices with Ki67 (a marker that does not distinguish between apical and basal progenitors) and an early marker of post-mitotic cells (Neun). As expected, we obtained a statistically significant increase in the number of Ki67/Neun double positive cells, confirming that cells that should be post-mitotic retain a proliferative identity for a longer period (Fig. 3.8D).

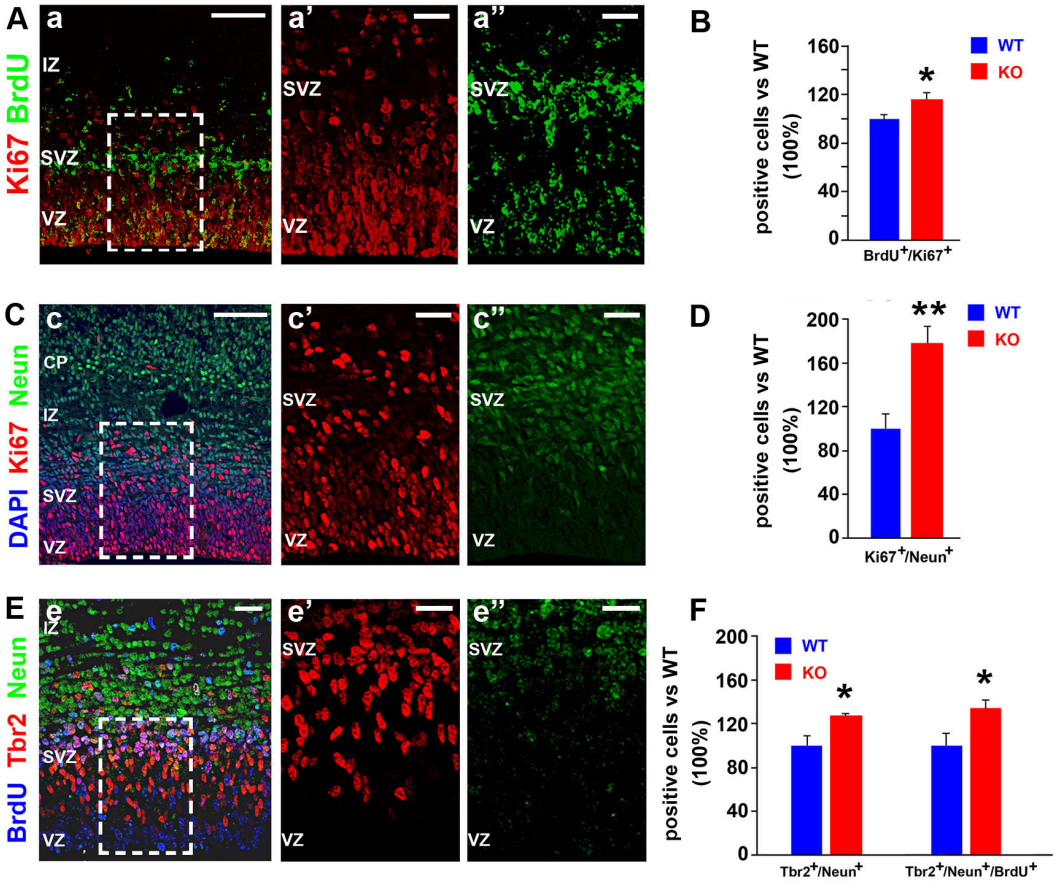

Figure 3.8. Lack of Mecp2 alters the number of cells transitioning from one developmental stage to the next one. A: the percentage of cells expressing the cell cycle marker Ki67 (**a'**) (synchronized with the BrdU injection; **a''**) is increased in ko samples (**B**). **C:** the increment of the Ki67/BrdU positive cells is possibly explained by the increment of cells transitioning from the mitotic to the post-mitotic condition (Ki67/Neun double positive cells (**c'**, **c''**), Neun defines post-mitotic neurons; **D**). **E:** accordingly, the switch between basal progenitors to neurons is altered (**e**), as the number of the double positive cells is increased (**F**). The images displayed in **a**, **c** and **e** represent the expression of the different markers in wt animals. Roughly 500 cells (counted in a binned grid) were counted per animal according to Ki67, BrdU or Neun immunoreactivity. Scale bar: 25 µm. Values are expressed as percentage versus wt ± the SEM (n=9 wt and 6 ko). Statistical analysis: Student's t test *= $p<0.05$; **=$p<0.01$.

To reinforce these observations, we analysed the progression leading from basal progenitors to Neun positive post-mitotic cells. We did not analyse the transition of apical (Pax6 positive) progenitors given these produce an overall low number of post-mitotic cells (through direct neurogenesis; (Attardo et al., 2008). Stunningly, the number of Tbr2 and Neun double positive cells resulted increased in ko samples compared to wt (Fig. 3.8F). Moreover, even the number of Pax6/Tbr2/BrdU triple positive cells (BrdU was administered, again, 24 hours before tissue collection) resulted increased, further confirming in ko cortices the increased size of the population of cells presenting an identity that is yet not completely defined (Fig. 3.8F).

Taken together, these *in vivo* data lead us to the hypothesis that a delay in the switch from one identity to the next one affects *Mecp2* null neuronal progenitors independently whether they are switching from one type of progenitors to the other (Fig. 3.7) or from progenitors to immature neurons (Fig. 3.8).

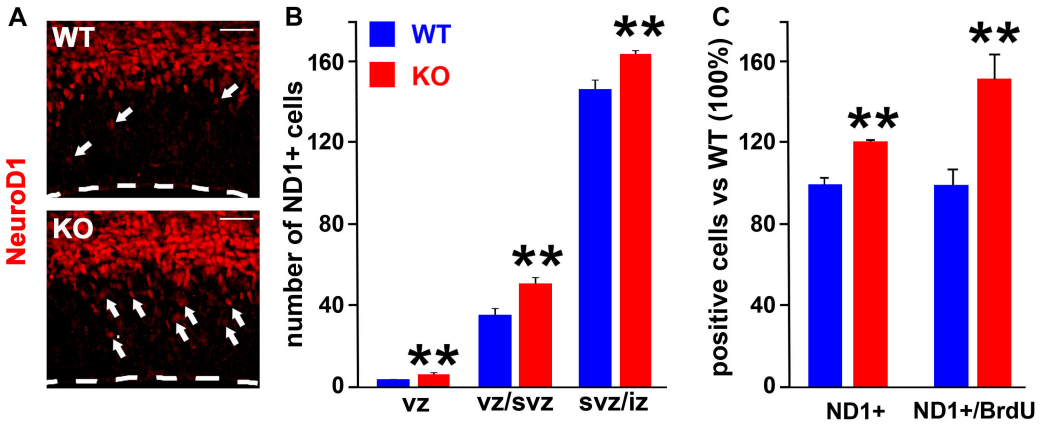

Figure 3.9. The number of *Mecp2* ko ND1$^+$ cells is increased compared to wt. ko samples display a rather large population of ND1$^+$ cells ectopically located within the VZ; accordingly, the number of such cells resulted increased in the SVZ and IZ (**A, B**). In **C** the overall increment of ND1$^+$ cells is represented. Scale bar: 50µm Values are expressed as percentage versus wt ± the SEM (n=6 wt and 6 ko). Roughly 500 cells (counted in a binned grid) were counted per animal according to ND1 staining. Statistical analysis: Student's t test *= $p<0.05$; **=$p<0.01$.

However, our evidences were produced exploiting markers that differentiate mitotic and post-mitotic populations; the NeuroD1 marker would define a transient stage that lasts from the latest phases of cell cycle to the earliest post-mitotic life of a newly

born neuron. We thus counted NeuroD1 positive cells in both wt and ko cortices at E15.5. In line with our hypothesis, we found an increment of NeuroD1 positive transitioning cells (Fig. 3.9A, B) that is even more evident when counted within the pool of BrdU (24 hours) positive cells.

Importantly, we believe that our results suggest that in the absence of Mecp2, in the neuronal lineage, cells express ectopic markers because they need more time to define their next phenotype; this impairment will lead to a delayed expression of genes characterizing more mature neurons, as demonstrated by our microarray analysis (Fig. 3.2C-E). We thus focused on characterizing the possible consequences of this defect beginning from the three main classes of genes that resulted deregulated in null samples from the analysis of the microarray data: glutamatergic receptors, ionic channels and intracellular signalling (Fig. 3.3B). The transcription of many ionotropic (AMPA and NMDA) and metabotropic glutamatergic receptors subunits was analysed through quantitative PCR (Fig. 3.10A) at different time points. We found that AMPA receptors subunits transcripts were mostly down-regulated starting from E15.5 through E18.5; such effect persisted even at post-natal levels (P8). NMDA subunits levels were mostly normal at E15.5 and, depending on the subunit analysed, start to be deregulated later on. Lastly, some subunits of the metabotropic receptors resulted down-regulated only at P8. This gradient of expression is most likely ascribable to the different timing in the activation of the transcription of these receptors in the developing cortex, accordingly, we were not able to detect any transcription of metabotropic receptors at E15.5.

Next, we analysed the expression of different ion channels subunits (Ca^{2+}, Cl^{-}, K^{+} and Na^{+}), which begins at E15.5 and persists later on (Fig. 3.10B). The deregulation of these membrane molecules is accompanied by the down-regulation of intracellular effectors, such as *Gnaq, Adcy9, Camk2β*, *Camk2d* and *Plcg2* (all molecules involved in intracellular signalling). Remarkably all these transcripts resulted mostly down-regulated at E15.5 and E18.5, and, for the most part, their expression was not renormalized by P8 (Fig. 3.10C).

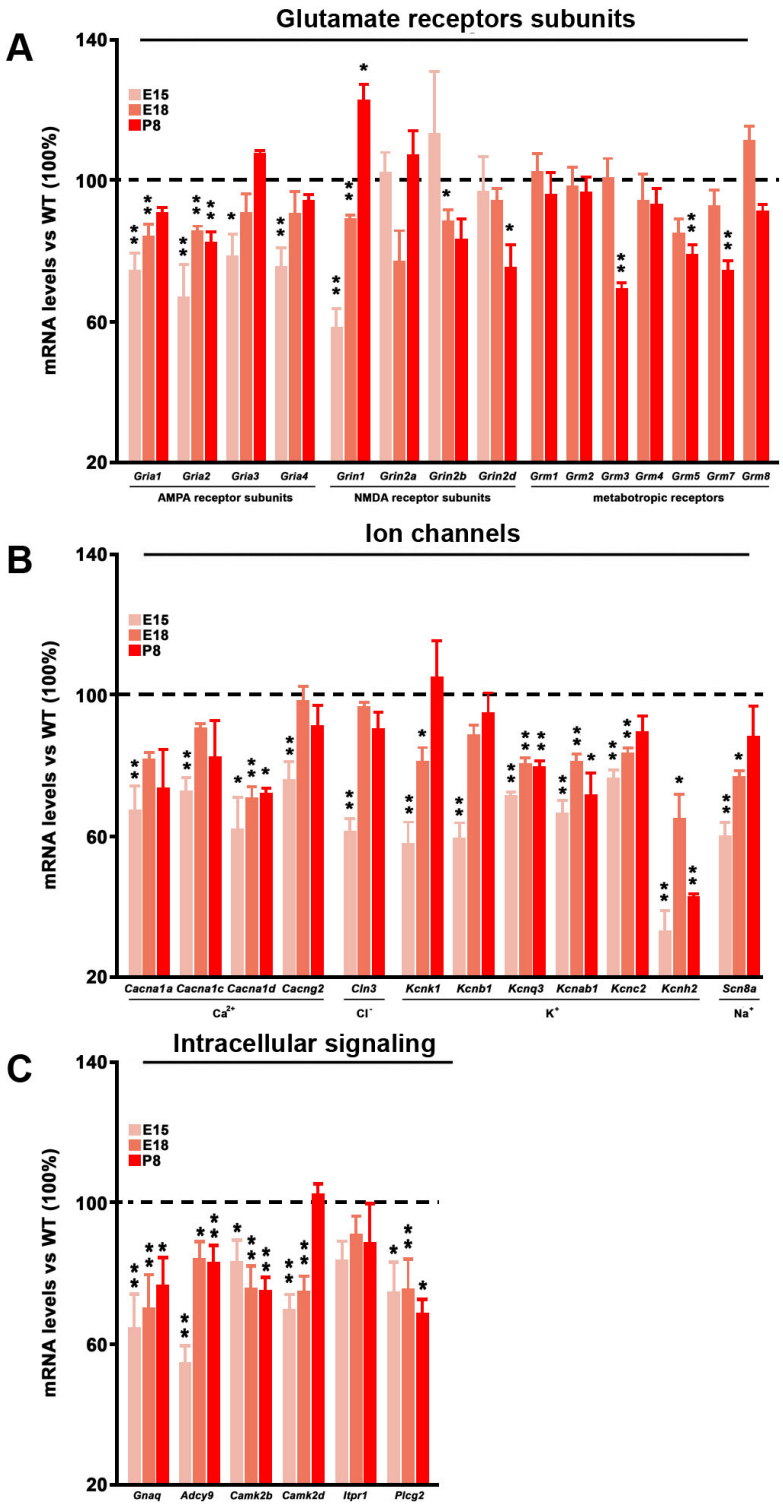

Figure 3.10. The expression of genes characterizing maturing/mature neurons is altered in the *Mecp2* ko. The qPCR analysis of cortices lacking Mecp2 displays the transcriptional deregulation of subunits composing glutamatergic receptors (**A**) and ion channels (**B**) and different components of intracellular signalling (**C**). Values are expressed as percentage versus wt ± the SEM (n =4 wt vs. ko in **A** and **B**; n =8 wt vs. 8 ko in **C**). Statistical analysis: Student's t test *= p<0.05; **=p<0.01 (Bedogni et al., 2015).

Interestingly, the expression of this group of genes characterizes maturing/mature electrically active neurons integrating (or integrated) in functional networks and reacting to external stimuli with proper intracellular responses. Given the mentioned deregulation of many genes involved in cortical excitability, we investigated whether neurons from ko animals show any impairment in their response to external stimuli.
Thus, we produced neuronal cultures from E15.5 cortices of wt and ko animals and evaluated cellular responsiveness to chemical (glutamate) and electrical stimulation in terms of calcium transients. At first, we characterized the responses of neurons to glutamate in wt conditions at very early stages of development (DIV3) by stimulating neurons with 25 μM glutamate. To block any effect triggered by stimuli other than glutamate, we added to the cultures the following blockers: Tetradotoxin (TTX to block Na^+ currents), Nifedipine (to block voltage gated Ca^{2+} channels) and Bicucullin (to block GABAergic receptors) respectively. In such conditions glutamate drove the strong Ca^{2+} wave depicted in figure 3.11a. To dissect whether such effect was mostly driven by AMPA or NMDA receptors, we first blocked AMPA receptors with CNQX (Fig. 3.11a'). Interestingly, AMPA receptors mediated responses after glutamate stimulation resulted by far the most predominant, as depicted in the graph in figure 3.11a'''. The fact that at this stage of maturation AMPA and NMDA are the only receptors mediating glutamate responses is demonstrated by the lack of responses driven by the addition of both CNQX and APV (NMDA blocker) to the medium (Fig. 3.11a''). To test responsiveness after field electrical stimulation (2 seconds at 30 Hz), we added CNQX, APV and Bicucullin to exclusively evaluate evoked calcium transients mediated by ionic channels (Fig. 3.11b). In such conditions we mostly abolished such responses by adding Nifedipine to the medium (roughly 75%; Fig. 3.11 b'), while no Ca^{2+} waves could be recorded after the administration of TTX alone (Fig. 3.11b''). These demonstrates that the calcium transients are mainly mediated by L-type Ca^{2+} channels (Fig. 3.11b''') and that their activation is mediated by the activation of Na^+ channels.

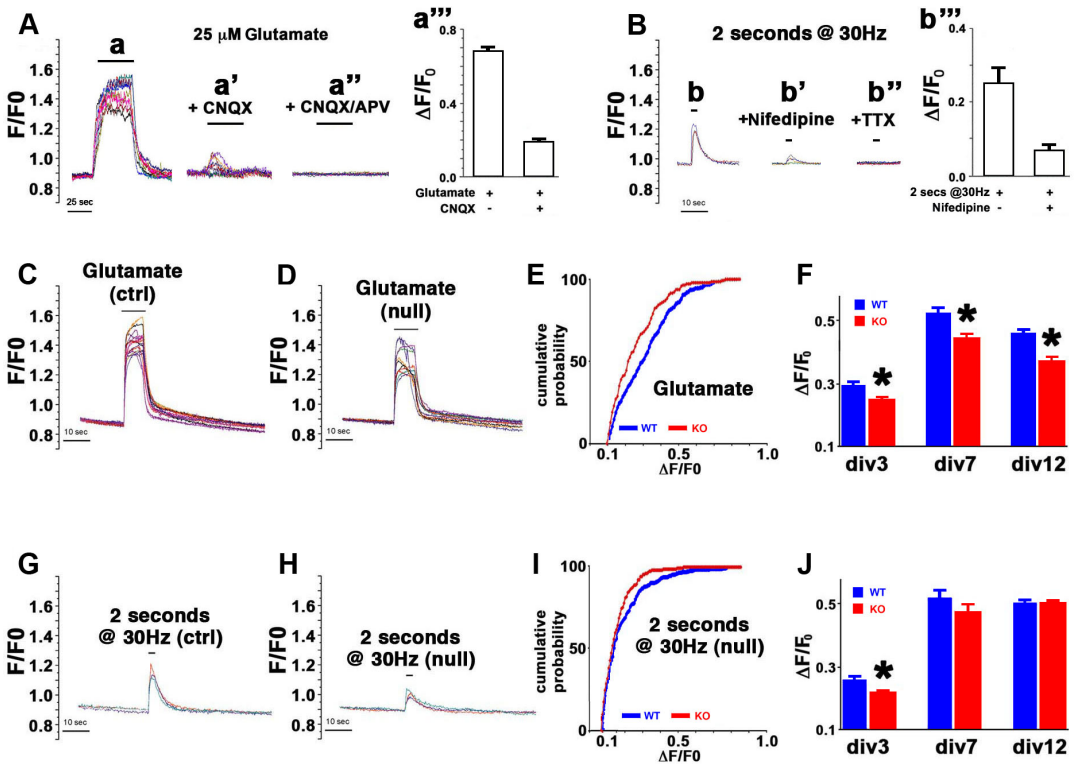

Figure 3.11. ***Mecp2*** **ko neurons exhibit lower calcium responses upon both glutamate and electrical stimulation.** Glutamate stimulation (25 μM) of neurons generated a strong calcium response (**a**) that is strongly reduced by adding 20 μM CNQX (**a'**) and completely abolished by adding 20 μM CNQX and 50 μM APV (**a''**), indicating that these responses are for the most part ascribable to AMPA receptors (**a'''**). Electrical field stimulation (2s at 30 Hz) generates a calcium response (**b**) that is strongly reduced by 20 μM Nifedipine (**b'**) and completely abolished by 1 μM TTX (**b''**), indicating that such response is mainly due to the activation of L-type Ca^{2+} channels, mediated by the activation of Na^{+} channels (**b'''**). The analyses of calcium changes upon either glutamate (**C–F**) or electrical stimulation (**G–J**) were performed on wild type and *Mecp2* ko neurons at different DIV (**F, J**) and resulted reduced at all the examined DIV fro the glutamate and at DIV3 for the electrical stimulation (**E, I:** cumulative analysis of DIV3 neurons). **F, J:** data represent the mean value of the ratio between ΔF and F0 at different time points. Values are expressed as the mean ± the SEM (n=4 wt and 3 ko). Statistical analysis: Student's t test *= $p<0.05$ (Bedogni et al., 2015).

In such conditions, we next studied the responses generated by either glutamate or electrical stimulation of DIV3, 7 and 12 wt and ko neurons. As depicted in figure 3.11C-F, glutamate (25 μM in presence of Nifedipine, TTX and Bicucullin) evoked responses were reduced in *Mecp2* null neurons at all the analyzed time points, therefore indicating a persistence of the impaired responsiveness. Interestingly, even when stimulated with field electric potentials (2 seconds at 30 Hz), ko neurons responses were reduced with respect to wt; however, such reduction was detected at DIV3 but not at later time points. We suggest that all in all these evidences indicate

that *Mecp2* null cortices are delayed in their ability to produce responses when stimulated probably because of their incomplete transition towards a more mature identity.

Whether this could affect not only the functionality by which *Mecp2* null neurons respond to stimuli but their actual structural maturation is hard to predict, however, the morphological analysis of ko neurons cultivated in vitro for only 3 days produced stunning evidences. In fact, we found that already at this early stage of development the nuclear size of ko neurons is reduced (Fig. 3.12A) and their long neurites (longer than 100 µm; Tuj1+) are shorter (Fig. 3.12B; no effects were detected on neurites shorter than 100 µm; Fig. 3.12C). This result is of extreme interest as it suggests that maturation defects driven by the lack of Mecp2 affect neuronal morphology ever since early development.

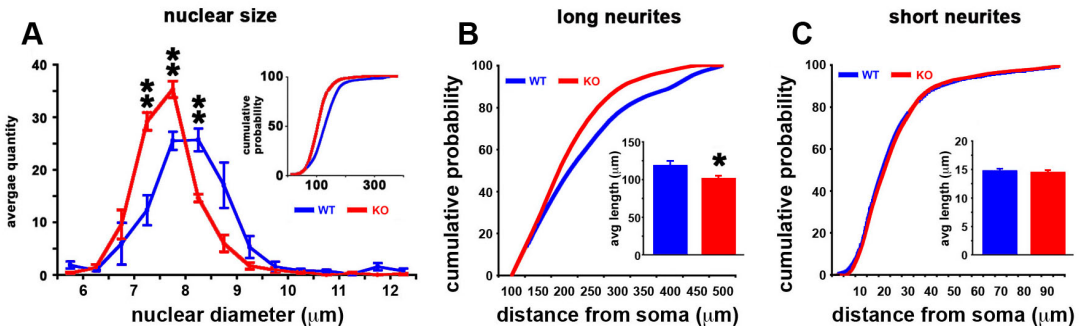

Figure 3.12. Morphological defects of DIV3 neurons lacking Mecp2. Already at such an early time points (DIV3) the nuclear size of ko neurons resulted reduced compared to wt (**A**). Moreover, the long neurites (longer that 100 µm) are shorter in ko neurons (**B**), while no effect is detectable in short neurites (shorter than 100 µm; **C**). Statistical analysis: Student's t test *= p<0.05; **=p<0.01. Kolmogorov–Smirnov testing revealed a p <0.001 for the cumulative analysis in **A** and <0.05 for the one in **B**, while resulted not significant for the one in **C**. n=3 ko and 4 wt (Bedogni et al., 2015).

4. DISCUSSION

Ever since the discovery of the link between MeCP2 mutations and RTT (Amir et al., 1999), many efforts in the field focused on identifying the physiological functions of the protein, in order to possibly restore them and ameliorate the conditions of the affected girls. One of the main characteristics of classical RTT is that females appear normal at birth, while overt symptoms arise during the first infancy (around 6-18 months of life) in a developmental phase that coincides with the peak of synaptogenesis in babies (Smeets et al., 2012). This evidence, together with the demonstration of the central role of the brain in RTT pathogenesis (Chen et al., 2001; Guy et al., 2001), suggested that RTT is a neurodevelopmental disorder, mainly affecting post-natal development. Indeed, it was demonstrated that Mecp2 has a role in proper neuronal arborisation (Armstrong, 2005) and in controlling synaptic activity, through the regulation of the initial formation of synaptic contacts (Chao et al., 2007). Nevertheless, earlier developmental phases might be impaired in RTT, as a growing number of evidences demonstrated that RTT patients and mice manifest subtle, but consistent, signs of the pathology also during the so called "pre-symptomatic" phase immediately after birth (Nomura, 2005; Picker et al., 2006), leading us to hypothesize that Mecp2 may have a role during embryonic development.

In the past, as previously mentioned, only few studies addressed the possible role of Mecp2 in embryos, but these did not lead to conclusive evidences (Kishi and Macklis, 2004; Schmid et al., 2008; Shahbazian et al., 2002). To shed light on this issue, we investigated the role of Mecp2 during early cerebral cortex development. The choice of studying cerebral cortex resided in the fact that this area is particularly involved in RTT pathogenesis; RTT patients, in fact, display cortical morphological abnormalities and strong cognitive impairments, often accompanied by seizures, which are mainly due to cortical dysfunctions (Armstrong, 2005).

The embryonic cerebral cortex is composed by heterogeneous cell populations that can be easily distinguished according to their maturation stage that is strictly linked to their positioning within the cortical wall and to the expression of specific

transcription factors. The 6-layered mature cortex arises from processes that are temporally and spatially defined ever since the establishment of Neuroepithelial Cells (NECs), composing the primordial dorsal telencephalon; such cells give raise to all the glutamatergic neurons composing the adult cortex (Fig. 4.1A). With the onset of neurogenesis (roughly at E11), NECs switch their mode of mitotic division from symmetric/proliferative to asymmetric/neurogenic, thus becoming Radial Glial Cells (RGCs; or Apical Progenitors, AP).

RGCs are not located within the entire cortical wall, as while neurogenesis proceeds and the embryonic cortex thickens, they remain in an apical area (i.e. close to the ventricle) that is defined as Ventricular Zone (VZ). The asymmetric division of these progenitors give rise to one RGC and either directly to a neuron or to a second type of progenitor, known as Intermediate Progenitor Cell (IPCs; or Basal Progenitor (BP), i.e. closer to the pia). IPCs are multipolar progenitors that symmetrically divide in the Sub-Ventricular Zone (SVZ) of the cortex. These cells can enter the cell cycle once, giving rise to two neurons, or twice, originating first two new IPCs that then generate neurons, thus exponentially expanding the neuronal pool (Farkas and Huttner, 2008; MuhChyi et al., 2013).

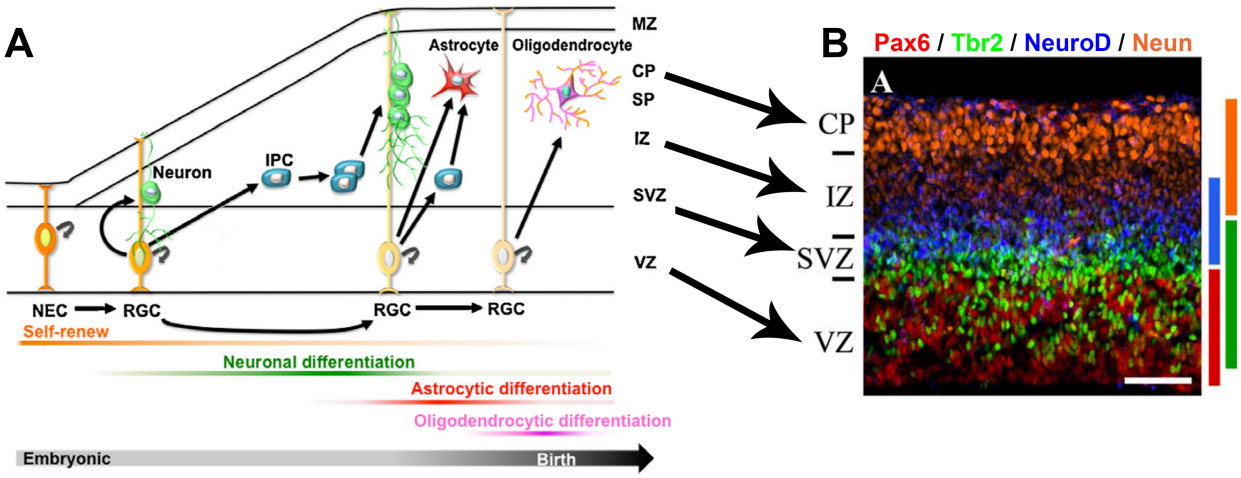

Figure 4.1. The development of the cerebral cortex. The cerebral cortex arises from the dorsal telencephalon that at E10 is formed by NECs symmetrically cycling to expand the progenitorial pool. Subsequently, NECs switch their division mode to asymmetric/neurogenic, becoming RGCs. Neurons are produced directly by RGCs or by IPCs that, dividing symmetrically, exponentially expand the neuronal production (**A**). Each cellular type populating the cortex is easily recognizable thanks to the expression of specific markers (**B**): Pax6 by RGCs, Tbr2 by IPCs, late mitotic/early post-mitotic cells by ND1, neurons by Neun (modified from MuhChyi et al. 2013; Hevner et al. 2006).

Interestingly, all the populations described are easily distinguishable in the cortical wall thanks to the expression of specific markers: RGCs express Pax6 and IPCs Tbr2. The progression through the next phases of neurogenesis requires the temporally regulated expression of NeuroD1, identifying cells switching from a progenitorial stage to a post-mitotic one, and Neun identifying early and late born neurons (Fig. 4.1B) (Hevner et al., 2006).

Given that all these are transcription factors, it is quite evident that a finely tuned transcriptional mechanism regulates the progression through the different phases enabling the production of post-mitotic neurons. The first wave of post-mitotic neurons migrates radially towards the pial surface forming the Pre-Plate (PP); the PP is then split into two layers (the Marginal Zone and the Subplate; MZ and SP) by a second wave of post-mitotic neurons that settle in between the PP giving rise to the Cortical Plate (CP, surrounded apically by the SP and basally by the MZ).

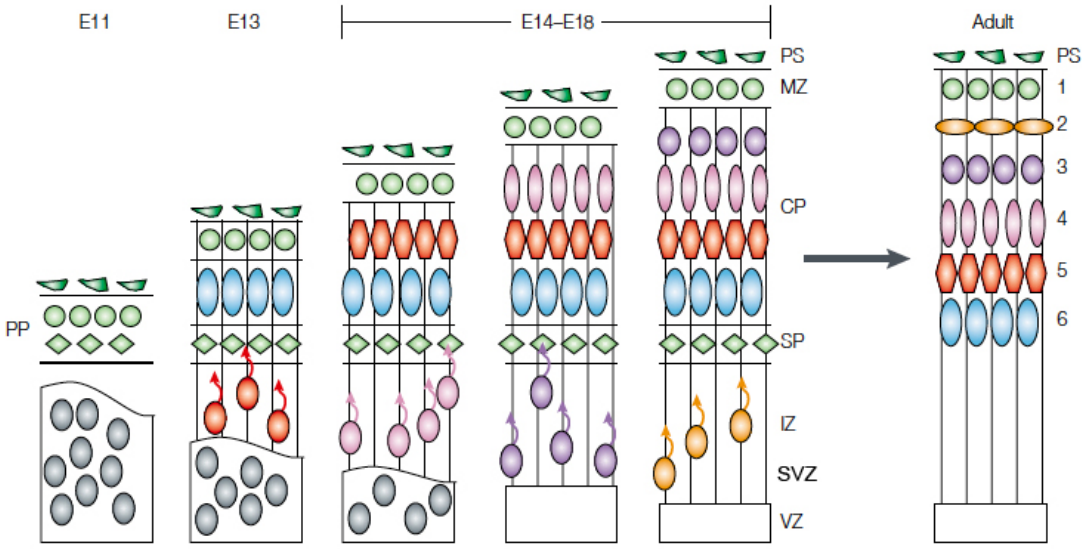

Figure 4.2. The 6-layered cerebral cortex is formed following an inside-out fashion. Neurogenesis begins in mice roughly at E11 and comprises different waves of production of post-mitotic neurons. These waves of neurons are produced in a specific temporal sequence and migrate towards the pia forming the 6-layered cortex following an inside-out dynamic: the oldest neurons form the inner layer and the youngest form the most superficial ones (Gupta et al., 2002).

All the subsequent waves of newly born neurons will migrate radially through the Intermediate Zone (IZ) in an inside-out fashion (Fig. 4.2): the oldest neurons form

the inner layers (L6-5-4-3 respectively) and the youngest cells more superficial ones (L2). The most superficial layer (L1) is the remnant of the MZ, in which CR cells reside. Immediately before birth, RGCs switch from the production of neurons/IPCs to the production of glial cells that continues post-natally (Gupta et al., 2002; Paridaen and Huttner, 2014).

We analysed the developing cortex at E15.5, as it represents the peak of corticogenesis. Moreover, at this stage only few GABAergic neurons have already migrated within the cortex and glial production is yet not started, thus the heterogeneity of the cellular population composing the E15.5 cortex is limited to progenitors and post-mitotic glutamatergic neurons. In this context, we demonstrated that all the cell types populating the cortex (progenitors, early and late post-mitotic neurons) express Mecp2, possibly suggesting that the protein exerts a function during the entire life of a neuron. Given the well-accepted role of Mecp2 in regulating transcription, we hypothesized that its absence could generate transcriptional derangements. Indeed, the transcriptional profile of E15.5 ko cortices display subtle, but consistent, alterations affecting many genes, rather than a few genes strongly deregulated. These results might reflect the hypothesized global function of Mecp2 (Skene et al., 2010). To extract meaningful information from this kind of dataset, it was thus necessary to analyse the enrichment of sets of genes (composed by transcripts sharing functional similarities or expressed by a common cellular type) rather than focusing on single, strongly deregulated, genes. Such approach enables to highlight the extent by which a "sum" of small transcriptional defects could be suggestive of the deregulation of an entire molecular mechanism (Bedogni et al., 2014; Subramanian et al., 2005). Through GSEA, that enables this kind of analysis, we obtained the enrichment of the proliferative (progenitorial) identity in the ko sample and the enrichment of the post-mitotic (neuronal) identity in the wt samples, suggesting that neuronal maturation in the ko cortex may be delayed. This result represents a first hint that already at early embryonic stages Mecp2 deficiency can produce slight but significant derangements in the mechanisms regulating the timing of the transition from cell cycle to post-mitotic differentiation.

Neurogenesis is based on a strict cross talk between cell division and differentiation; since these two processes are closely coordinated, defects in either one mechanism

could deeply alter the establishment of a functional network. Given the aforementioned delay by which the post-mitotic identity is acquired, it is very likely that the early mitotic steps required for the establishment of cortical network is impaired in the *Mecp2* null embryonic cortex. We thus searched for any possible alteration of the rate of cell cycle progression and the balance between cell cycle re-entry and exit in *Mecp2* null samples (Dehay and Kennedy, 2007).

We demonstrated that the cell cycle progression is slightly delayed in ko NPCs due to impairments affecting the time window covered by the G1 phase, both *in vitro* and *in vivo*. This is of particular interest, as the G1 phase is crucial in determining whether a progenitor will re-enter the cell cycle or rather differentiate. Indeed, a precise step in G1, known as "restriction point", defines when the cell is definitively committed to re-enter the cell cycle; after this point, a transcriptional mechanism enabling the transition from G1 to S phase is activated reinforcing, through a positive feedback, the cell cycle progression that then proceeds independently from external cues (Bertoli et al., 2013). It is well known that the length of G1 increases during late steps of neurogenesis compared to early ones (Arai et al., 2011) possibly enabling the progenitors to respond to external stimuli and to accumulate different factors leading to either cell cycle exit or re-entry (Hardwick et al., 2014). The alterations encountered by *Mecp2* null cells in G1 phase may be thus suggestive of a problematic switch from proliferation to differentiation.

In light of this, we next verified whether the previously demonstrated delayed progression through the cell cycle alters neuronal maturation *in vivo*. Interestingly, our data show, in the null samples, a statistically significant increase of the cells co-expressing markers that normally are progressively switched on and off by a cell during its transition from RGC to IPC ($Pax6^+ \rightarrow Tbr2^+$; Fig. 4.3). This does not imply that the *Mecp2* null cortex displays an increased total number of either RGCs or IPCs but, rather, that the fate of such cells is not as sharply defined as in wt samples, with IPCs retaining some RGCs identity. Accordingly, we found an increased number of KI67/Neun and Tbr2/Neun double positive cells, demonstrating that part of the progenitorial identity is still featured by post-mitotic neurons; of note, these results well match with the increased representation of proliferative identity highlighted through GSEA. The increased number of cells expressing markers characterizing two

different populations could imply that Mecp2 deficiency causes a defect in defining the identity of a cell. As mentioned, during the development of the cerebral cortex the sequential expression of transcription factors generates a "combinatorial code" that is fundamental to specify the final cellular fate (Englund et al., 2005; Hevner et al., 2006). Thus, the altered G1 phase may cause the persistence of a slightly unrefined identity that may delay the progression towards a fully matured stage, rather than affecting neurogenesis in terms of number of neurons produced. This is in agreement with the fact that both in humans and animal models the gross morphology of the *MECP2* defective cerebral cortex and the average number of neurons composing it are not impaired (Armstrong, 2005; Kishi and Macklis, 2004).

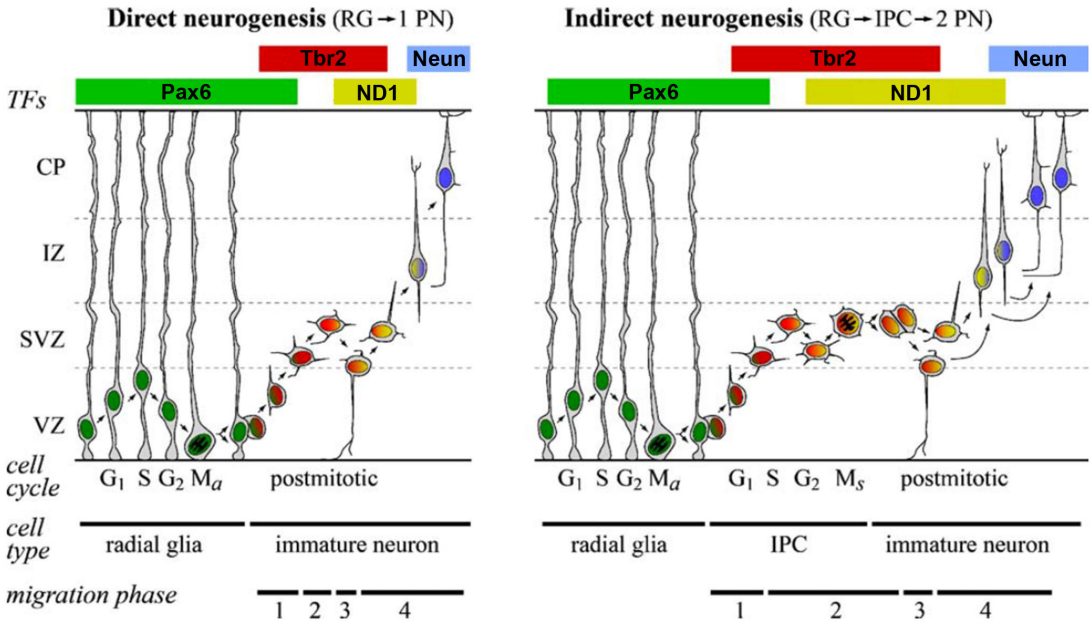

Figure 4.3. Cell maturation in the cerebral cortex is accompanied by the sequential expression of transcription factors. These markers are co-expressed in a cell for restricted time lapses, during the switch of their identity (modified from Hevner et al. 2006)

Accordingly, a similar phenotype was previously reported by Smrt et al, demonstrating that, during adult neurogenesis in the hippocampus, *Mecp2* null samples display an increased number of transitioning neurons switching from immature stages to mature ones, suggesting that cells lacking Mecp2 fail in properly differentiating towards established neurons (Smrt et al., 2007). By unmasking an increased number of cells expressing ND1, we further confirmed the increment of

transitioning cells in null samples. Indeed, ND1 is a marker of late mitotic/early post-mitotic phases that enables to identify cells switching from proliferation to differentiation.

Overall, the impaired transition between mitotic and post-mitotic identity may be linked with the maturation impairments previously suggested by our transcriptional analysis highlighting the enrichment of the identity of the post-mitotic compartment in the wt samples. Indeed, we demonstrate that *Mecp2* null cortices are characterized by the down-regulation of different glutamate receptors and ion channel subunits, transcripts that are mostly expressed by maturing post-mitotic neurons. Such defects are involved in the reduced capability of neurons to respond to external stimuli such as glutamate or current injection, as demonstrated by calcium imaging analysis. In accordance with our findings, it is known that the establishment of networks is impaired when Mecp2 is absent (Dani et al., 2005) and there is a strict link between externally driven responses and neuronal arborisation (Spitzer, 2006). Importantly and new for the field, we demonstrated that in the earliest phases of maturation, *Mecp2* null neurons display morphological alterations that are typically displayed by null neurons in adulthood (reduced nuclear size and shorter neuritis). A link between such early morphological and functional phenotype and the deranged transition between proliferative and post-mitotic phases could be easily hypothesized: cells encountering problems in defining their identity are possibly delayed in their subsequent maturation. Interestingly, this could also directly affect the proliferative compartment itself; in fact, it is known that post-mitotic neurons influence the rate of proliferation/differentiation of progenitors residing in the VZ and SVZ thanks to feedback mechanisms (through descending axons) (Dehay and Kennedy, 2007). Given the known problems in synaptogenesis, this mechanism could very likely be affected in null samples.

To conclude, we demonstrate that during cortical development lack of Mecp2 affects cell fate decision most likely delaying post-mitotic neuronal maturation; this is possibly mediated by transcriptional deregulations delaying the acquirement of a more mature identity by turning off the previous one. This delay could drive, beside other effects, the reduced expression of membrane molecules necessary to respond to

external stimuli, thus reducing cell responses to the environment delaying the integration of immature neurons in a fully established network.

We believe that RTT phenotype is thus the sum of different alterations occurring ever since embryonic developmental stages that worsen during early infancy, generating the overt establishment of symptoms.

Our hypothesis of a role for Mecp2 during cell differentiation is indeed reasonable, as stem cells, differentiating to more specialized phenotypes, progressively restrict their fate by repressing specific genes. This transcriptional regulation is, at least partially, ascribable to epigenetic changes that modify the chromatin structure and fold chromosomal regions into non-transcriptionally active heterochromatin. Since epigenetic mechanisms are involved in this process, it is possible to infer that lack of an epigenetic factor such as Mecp2 can generate impairments in the interpretation of the "methylation signature" characterizing the transcriptional identity that specifically define each cell type (MacDonald and Roskams, 2009; MuhChyi et al., 2013).

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Review

Rett syndrome and the urge of novel approaches to study MeCP2 functions and mechanisms of action

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ABSTRACT

Rett syndrome (RTT) is a devastating genetic disorder that worldwide represents the most common genetic cause of severe intellectual disability in females. Most cases are caused by mutations in the X-linked *MECP2* gene. Several recent studies have demonstrated that RTT mimicking animal models do not develop an irreversible condition and phenotypic rescue is possible. However, no cure for RTT has been identified so far, and patients are only given symptomatic and supportive treatments. The development of clinical applications imposes a more comprehensive knowledge of MeCP2 functional role(s) and their relevance for RTT pathobiology. Herein, we thoroughly survey the knowledge about MeCP2 structure and functions, highlighting the necessity of identifying more functional domains and the value of molecular genetics. Given that, in our opinion, RTT ultimately is generated by perturbations in gene transcription and so far no genes/pathways have been consistently linked to a dysfunctional MeCP2, we have used higher-level bioinformatic analyses to identify commonly deregulated mechanisms in MeCP2-defective samples. In this review we present our results and discuss the possible value of the utilized approach.

Contents

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Methyl-CpG Binding Protein 2 (MeCP2) Localizes at the Centrosome and Is Required for Proper Mitotic Spindle Organization*[S]

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Background: MeCP2 is a multifunctional protein whose full spectrum of activities remains enigmatic.
Results: MeCP2 localizes at the centrosome and at the mitotic spindle. Its loss causes deficient spindle morphology and microtubule nucleation and prolonged mitosis.
Conclusion: Through its centrosomal localization, MeCP2 regulates cell growth and cytoskeleton stability.
Significance: This novel MeCP2 function may improve our comprehension of MeCP2 under physiological and pathological conditions.

Mutations in *MECP2* cause a broad spectrum of neuropsychiatric disorders of which Rett syndrome represents the best defined condition. Both neuronal and non-neuronal functions of the methyl-binding protein underlie the related pathologies. Nowadays MeCP2 is recognized as a multifunctional protein that modulates its activity depending on its protein partners and posttranslational modifications. However, we are still missing a comprehensive understanding of all MeCP2 functions and their involvement in the related pathologies. The study of human mutations often offers the possibility of clarifying the functions of a protein. Therefore, we decided to characterize a novel MeCP2 phospho-isoform (Tyr-120) whose relevance was suggested by a Rett syndrome patient carrying a Y120D substitution possibly mimicking a constitutively phosphorylated state. Unexpectedly, we found MeCP2 and its Tyr-120 phospho-isoform enriched at the centrosome both in dividing and postmitotic cells. The molecular and functional connection of MeCP2 to the centrosome was further reinforced through cellular and biochemical approaches. We show that, similar to many centrosomal proteins, MeCP2 deficiency causes aberrant spindle geometry, prolonged mitosis, and defects in microtubule nucleation. Collectively, our data indicate a novel function of MeCP2 that might reconcile previous data regarding the role of MeCP2 in cell growth and cytoskeleton stability and that might be relevant to understand some aspects of MeCP2-related conditions. Furthermore, they link the Tyr-120 residue and its phosphorylation to cell division, prompting future studies on the relevance of Tyr-120 for cortical development.

Methyl-CpG binding protein 2 (MeCP2) was first described as an epigenetic factor that binds specifically to methylated DNA and represses transcription through the recruitment of chromatin remodeling complexes containing histone deacetylase activities (1, 2). Mutations in the X-linked *MECP2* gene were later found in several patients affected by Rett syndrome (RTT,[4] OMIM no. 312750), a devastating neuronal disease that, because of its incidence, is considered to be one of the main causes of severe intellectual disabilities in girls (3). Since then, hundreds of different mutations in *MECP2* have been associated with RTT or, less frequently, with other forms of intellectual disability.

Although *MECP2* mutations have profound effects on brain functions, several recent studies have demonstrated that RTT is not an irreversible condition in mice because phenotypic rescue is possible (4). MeCP2 studies have therefore been boosted dramatically, leading to a progressive expansion of MeCP2 functions beyond the original role of the protein in transcriptional repression through the recruitment of chromatin remodeling complexes (5). In 2003, Georgel *et al.* (6) proposed that MeCP2, when highly abundant, might work directly (without other corepressors or enzymatic activities) as a potent chromatin condensing factor . Accordingly, Skene *et al.* (7) have demonstrated that, in mature neurons where MeCP2 levels are sufficiently high to saturate methylated DNA but not in non-neuronal cells characterized by 10–30 times less MeCP2, the protein can substitute histone H1 and function as a global

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[4] The abbreviations used are: RTT, Rett syndrome; PTM, posttranslational modification; MEF, mouse embryonic fibroblast; λ-PPase, λ-phosphatase; WB, Western blot.

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Original Article

ORIGINAL ARTICLE

Defects During *Mecp2* Null Embryonic Cortex Development Precede the Onset of Overt Neurological Symptoms

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Abstract

MeCP2 is associated with several neurological disorders; of which, Rett syndrome undoubtedly represents the most frequent. Its molecular roles, however, are still unclear, and data from animal models often describe adult, symptomatic stages, while MeCP2 functions during embryonic development remain elusive. We describe the pattern and timing of *Mecp2* expression in the embryonic neocortex highlighting its low but consistent expression in virtually all cells and show the unexpected occurrence of transcriptional defects in the *Mecp2* null samples at a stage largely preceding the onset of overt symptoms. Through the deregulated expression of ionic channels and glutamatergic receptors, the lack of Mecp2 during early neuronal maturation leads to the reduction in the neuronal responsiveness to stimuli. We suggest that such features concur to morphological alterations that begin affecting *Mecp2* null neurons around the perinatal age and become evident later in adulthood. We indicate MeCP2 as a key modulator of the transcriptional mechanisms regulating cerebral cortex development. Neurological phenotypes of *MECP2* patients could thus be the cumulative result of different adverse events that are already present at stages when no obvious signs of the pathology are evident and are worsened by later impairments affecting the central nervous system during maturation and maintenance of its functionality.

Key words: embryonic cerebral cortex development, intrinsic neuronal excitability, MeCP2 null cortex, neurological disorders, transcriptome

Introduction

Rett syndrome (RTT) is a devastating genetic disorder that worldwide represents the most common genetic cause of severe intellectual disability in females. Typical RTT patients appear to develop normally throughout the first 6–18 months of life when neurological development arrests and a regression phase leads to the loss of previously acquired skills. During and after the regression phase, patients develop a host of typical symptoms

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Abstract:	MECP2 mutations cause a broad spectrum of neurological disorders of which Rett syndrome (RTT) represents the best-defined condition. Several mouse models of RTT have been generated whose face validity is demonstrated by the presence of a broad spectrum of phenotypes largely mimicking those manifested by RTT patients. Importantly, these models have permitted to demonstrate that RTT is not an irreversible condition. Although much has been produced describing the different roles played by Mepc2, still much is needed to possibly propose translational studies. Mouse models are therefore crucial for the identification of valid therapeutic approaches. Since most MECP2 pathogenic lesions are considered loss of function mutations, the Mecp2-null alleles have construct validity; accordingly, the C57/B6 Mecp2tm1.1Bird line probably represents the mostly used Mecp2 model. However, small litters with little viability, under-representation of the mutant allele and poor maternal care hamper colony maintenance, and severely limit research. To ameliorate and facilitate managing the Mecp2 null colony, we have transferred the Mecp2tm1.1Bird allele on the stronger and easier-to-breed CD1 background. The obtained strain recapitulates several phenotypes described in mouse models of Mecp2, but does not present the breeding difficulties encountered with the C57/B6 strain. The validity of this strain is strengthened by longer lifespan and better general conditions of the null animals. Thus, we believe that the use of this validated mouse model has the potentiality to improve research on RTT.
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